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Characterization of NK cells in mouse models of Systemic Lupus Erythematosus and of the role of the p85β PI3K subunit in NKG2D signaling in NK cells

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Abbreviations

AEC – *3-Amino-9ehtylcarbazole*

ALPS- *Autoimmune Lymphoproliferative Syndrome*

APC- *Antigen Presenting Cell*

BM – *Bone Marrow*

BrDU - *5-bromo-2'-deoxyuridine*

BSA – *Bovine Albumin Serum*

CBL – *Casital B-lineage Lymphoma*

CD – *Cluster of Differentiation*

CHO - *Chinese Hamster Ovary*

CX3CL1 - *Chemokine (C-X3-C motif) ligand 1*

DNAM-1 – *DNAX accessory molecule 1*

EDTA – *Ethylenediaminetetraacetic acid*

ELISA – *Enzyme-Linked Immuno-Sorbent Assay*

FACS – *Fluorescence-Activated Cell Sorting*

FBS – *Fetal Bovine Serum*

G418 – *Geneticin*

Grb2 – *Growth factor receptor-bound protein 2*

HBS – *HEPES Buffered Saline*

HIER – *Heat Induced Epitope Retrieval*

HSC – *Hematopoietic stem cell*

ICAM1 – *Inter-Cellular Adhesion Molecule 1*

IF - *ImmunoFluorescence*

IFN-γ - *Interferon gamma*

IHC – *Immunohistochemistry*

IL-2 – *Interleukin 2*

IL-6 – *Interleukin 6*

IL-10 – *Interleukin 10*

IL-12 – *Interleukin 12*

IL-15 – *Interleukin 15*

IP-10 - *Interferon gamma-induced protein 10*

iNK – *Immature Natural Killer Cell*

ITAM – *Immunoreceptor Tyrosine-based Activation Motif*

JAK – *Janus Kinase*

KIR – *Killer-cell Immunoglobulin-like Receptor*

LIN – *Lineage*

LPR - *Lymphoproliferation spontaneous mutation*

Ly49 – *Killer cell lectin-like receptor subfamily 49*

MAPKs – *Mitogen Activated Protein Kinases*

MHC – *Major Histocompatibility Complex*

MULT-1 - *Murine UL16-binding protein-like transcript*

MRL – *Murphy Roths Large mouse*

MS – *Multiple Sclerosis*

NK – *Natural Killer Cell*

NK1.1 – *NK cell associated marker*

NKG2D - *Natural Killer Group 2D Receptor*

NKP – *Natural Killer Precursor*

O/N – *Overnight*

PBMC – *Peripheral Blood Mononuclear Cell*

PBS – *Phosphate Buffered Saline*

PFA - *Paraformaldehyde*

PI – *Propidium Iodide*

PI3K – *Phosphoinositide 3-kinase*

PMA - *Phorbol 12-myristate 13-acetate*

RA – *Rheumatoid Arthritis*

Rae – *retinoic acid early inducible gene 1*

RAG – *Recombination Activating Gene*

RANTES - *Regulated on Activation, Normal T cell Expressed and Secreted cytokine*

RMA/S - *Rauscher murine leukemia virus-induced tumors cell line*

RPMI – *Roswell Park Memorial Institute medium*

RT – *Room Temperature*

S2 – *Drosophila Schneider 2 Cells*

SLE – *Systemic Lupus Erythematosus*

STAT5 - *Signal Transducer and Activator of Transcription 5 protein*

T1D – *Type 1 Diabetes*

TCR – *T Cell Receptor*

Th-1 – *T-helper cell type 1*

Th-2 – *T-helper cell type 2*

TNF-α – *Tumor Necrosis Factor Alpha*

WT – *Wild type*

YAC-1 - *T cell lymphoma induced by inoculation of the Moloney leukemia virus (MLV) into a newborn A/Sn mouse*

Summary

Natural killer (NK) cells are large granular lymphocytes that play an important part as a link between the innate and adaptive immune systems. Various autoimmune diseases are correlated with alterations in the activity of NK cells; nonetheless, their role in the pathogenesis of systemic lupus erythematosus (SLE), a complex multi-factorial disease, has not been extensively studied. One of the most important activating NK cell receptors is NKG2D, which is able to signal via the p85 subunit (either the α or β subunit) of the PI3K (phosphatidylinositide 3-kinase) enzyme, to which it binds indirectly via the DAP10 adaptor molecule. It is not known which of the two p85 subunits binds preferentially to DAP10, or whether the p85β subunit participates in mediating NKG2D receptor signaling.

In the first part of this work, we studied the role of NK cells in murine models of SLE. We show that the MRL/MpJ mouse is a useful model for this analysis, and that NK cells in diseased mouse spleen suffer from impaired differentiation, which is site-specific. In addition, we show that NK cells in the kidneys of diseased SLE-like mice are phenotypically more mature and more active compared to those of healthy mice and in other non-target organs. We report the expression of the NKG2D ligands Rae-1 and Mult-1 specifically in the glomeruli of various lupus-prone mouse models. Expression of these ligands increases concomitantly with disease state and influences NK cell maturation and IFN-γ production.

For the second section of the study, we analyzed the PI3K p85β subunit function in NK cells. We demonstrate that p85β is not involved in NK cell differentiation, proliferation, or maturation. The p85β-deficient NK cell phenotype is more activated than that of control mouse NK cells, as seen via cytotoxicity assays and cytokine secretion. We found that this activated phenotype is probably due to the role of p85β in NKG2D internalization, which is impaired in as p85β-deficient NK cells.

Our findings identify an undescribed NK cell function in SLE pathogenesis and of the p85β PI3K subunit in the regulation of the NKG2D receptor function.

Resumen

Las células “asesinas naturales” (NK) son linfocitos que sirven como enlace entre el sistema inmune adaptativo e innato. Alteraciones en la actividad en las células NK están correlacionadas con enfermedades autoinmunes, aunque el papel de estas células en el Lupus Sistémico Eritromatoso (SLE), una enfermedad compleja y multifactorial, aún sigue siendo controvertido. Uno de los receptores de activación mas importantes en las células NK es el receptor NKG2D. NKG2D se une a la molécula adaptadora DAP10, y señaliza a través de la subunidad p85 del enzima PI3K. Aún no se sabe cual de las dos subunidades de p85 se une preferiblemente a la molécula adaptadora DAP10, ni si la subunidad p85β tiene un papel en la señalización mediada por el receptor NKG2D.

En la primera parte de este trabajo, hemos estudiado el papel de las células NK en modelos murinos de SLE. Demostramos que el modelo MRL/MpJ es un modelo válido para el estudio de células NK en SLE, y que las células NK en el bazo de ratones enfermos tienen defectos en diferenciación. Demostramos también que células NK en los riñones de ratones enfermos con SLE son fenotipicamente más maduras y más activas comparadas con las células NK de ratones sanos, y con las células NK presentes en órganos no afectados por la enfermedad. Además, reportamos la expresión de los ligandos de NKG2D Rae-1 y Mult-1 específicamente en los glomérulos de varios ratones con susceptibilidad genética a desarrollar SLE. La expresión de estos ligandos aumenta con la progresión de la enfermedad, y que la expresión de estos ligandos influye en la maduración y producción de IFN-γ por parte de células NK.

En la segunda parte de este trabajo hemos estudiado el papel de la subunidad p85β en células NK. Demostramos que la subunidad p85β no está involucrada en la diferenciación, proliferación, o maduración de células NK. Sin embargo, mostramos cómo las células NK de ratones deficientes de p85β tienen un fenotipo mas activado, como se demuenstra mediante ensayos de citotoxicidad y secreción de citoquinas. Este fenotipo más activo podría deberse al papel que p85β juega en la internalización de NKG2D, dado que las células NK deficientes en p85β tienen defectos en la internalización de dicho receptor.

Estos resultados indican un papel nuevo para las células NK en la patogénesis de SLE y de la subunidad p85β de PI3K en la regulación de las funciones del receptor NKG2D.

Introduction

If I have seen further it is by standing on the shoulder of giants
Sir Isaac Newton

The last thing one discovers in composing a work is what to put first
Blaise Pascal, Pensées

Part 1 – Natural Killer Cells – Functions and development

Natural Killer Cell functions and its receptors

Natural killer (NK) cells are large granular lymphocytes that constitute an essential element of the innate immune system, being after T and B cells, the third major lineage of lymphocytes [1]. NK cells were first characterized for their capacity to kill tumor targets without prior sensitization [2, 3]. NK cells are in fact well known for their capacity to lyse susceptible target cells via the exocytosis of perforin and granzyme granules, which are capable of inducing apoptosis in, targeted cells [4]. NK cells are also known to be rapid producers of various cytokines and chemokines such as IFN-γ, TNF-α, RANTES, MIP-1α/β which are capable of amplifying and recruiting an inflammatory response via various mechanisms [5, 6].

Their main distinction from T and B cells is that NK cells do not use the recombination-activating gene enzymes (RAG) for the rearrangement of their receptor genes and they do not possess a unique antigen recognition receptor [7, 8]. This NK cell specific characteristic is determined by their capacity to interact with virtually all nucleated cells, via a family of inhibitory receptors, either the Ly49 family in mice, or the killer cell immunoglobulin-like receptor (KIR) family in humans [9]. These receptors are capable of ascertaining if the cells with whom the NK cells interact express the correct self-MHC profile. The lack of a correct MHC-profile, usually via the lack of MHC class I molecules, does not allow for the binding of these inhibitory NK cell receptors, and thus allows for subsequent NK cell activation and lysis of the target cell. This was what was referred to as the "missing-self" hypothesis, and was specifically noted in MHC-I class deficient tumor cell lines [10].

Substantial evidence is now had that NK cells do not only possess inhibitory, but also activatory receptors **(Table I.1)**. Activatory receptors in NK cells include a myriad of different subsets, including the CD49/NKG2C complex, NKp46 and NKG2D (found in both mice and humans), Ly49 subsets (in mice), and the KIR S family in humans [11]. These activatory receptors are capable of recognizing both foreign and self-proteins. Indeed, some, such as Ly49H are capable of recognizing the cytomegaloviral MHC Class I-related molecule, others such as NKp46 bind viral haemagglutinins, and other receptors, such as NKG2D, bind self-molecules which are upregulated on the cell membranes of "stressed" or virally infected cells [12-14]. NKG2D activation correlates with that of NK cells, leading to the lysis of infected, stressed or cancer cells, both in vivo and in vitro [15, 16]. NKG2D is activated by NKG2D ligands, a "stress-induced" family of MHC-I-like proteins. Seven ligands are reported in humans (MICA, MICB, ULBP1-ULB4, RAET1G) and mice (Rae1α/β/γ/ε/δ, MULT-1, H60). This has led to a revised theory on the "missing self" hypothesis, in which an NK's activatory/quiescent state is struck by a balance of its

activatory and inhibitory receptors on its cell surface [17].

Receptor	Class	Motif/Adaptor	Ligand
Adhesion Receptor			
CD2 (LFA-2)	IgSF	Proline-rich domain	CD48, CD58
CD11a (LFA-1)	IgSF	Src family kinases, PI3K	CD54 (ICAM-1), CD107 (ICAM-2)
CD11b (MAC-1)	IgSF	?	CD54 (ICAM-1)
CD43	IgSF	?	E-Selectin (?)
CD49b (DX5)	IgSF	?	Collagen (?)
Activating Receptors			
CD69	C-lectin	?	?
CD122 (IL-2Rβ)	Cytokine Receptor	JAK1, 3; STAT5	IL-2, IL-15
NKG2D	C-Lectin	YINM/DAP10, PI3K	Rae1, Mult-1, H60
Ly49 D, H, P	C-Lectin	ITAM/DAP12	H-2 class I, MCMV
DNAM-1	IgSF	?	CD112
NK1.1	C-Lectin	FCεRIγ	?
NKp46	IgSF	?	Viral HA, HSPG
Inhibitory receptors			
NKG2A	C-lectin	ITIM/SHP-1	Qa1b
CD244	SLAM	TXYXXV-I/SAP	CD48
Ly49 A-C, E-G, I-O	C-lectin	ITIM/SHP-1, -2	H-2 class I

Table I.1 – List of the main adhesion, activating and inhibitory receptors of murine NK cells

Development of Natural Killer Cells

NK cells are known to develop primarily in the bone marrow (BM), and unlike T cells, do not require the thymus for their development, since various studies have reported normal numbers of NK cells in athymic nude mice [2, 3, 18]. NK cells, as is the case with all cells in the heamatopoietic system, are derived from BM heamatopoietic stem cells (HSC). Via a continuous

and sequential loss of pluripotency, HSCs differentiate into what are known as Natural Killer cell precursors (NKP). NKPs have been characterized in the BM and fetal thymus of mice with a $Lin^{-}CD122^{+}NK1.1^{-}DX5^{-}$ phenotype[1, 19, 20]. CD122 is a 70-75 kD IL-2 receptor β chain also known as IL-2Rβ, which is also shared by the IL-15 receptor[1, 21]. Its expression is crucial for the further development of NKPs, as it allows NKPs to respond to IL-15 stimulation[22]. IL-15 has been described as the main player in NK cell development in the bone marrow, as studies using IL-15 knockout mice are almost completely devoid of NK cells [23-25]. Furthermore, NKP cells stimulated with IL-15 are capable of proliferating and differentiating into mature NK cells with both cytotoxic and cytokine secretion capacities [1, 20]. Nonetheless, IL-15 is not the be-all and end-all of NK cell development. Indeed, even though IL-15 deficient mice were almost devoid of NK cells, the mere presence of residual NK cells, and the fact that these show some degree of maturation indicate that more cytokines and or factors play a role in NK cell arisal. [26-28]. Once formed, NKPs must progress into fully mature phenotypic and functional NK cells (mNK). This occurs via the continuous process of NKP maturation into immature NK cells (iNK). iNK cells are characterized via several markers which can distinguish differentiating NK cells. iNK cells still lack both the complete and functional attributes of mNK cells, and are defined via the progressive acquisition of NK cell markers such as CD122, NK1.1, DX5, NKG2D, CD27, CD43, and CD11b [29] **(Figure I.1)**.

NK cell trafficking

It was originally postulated that NKPs would arise in the BM, differentiate, and then start recirculating in the body. Indeed, evidence supports the idea that the BM is the primary site of NKP generation, seeing as how BM ablation drastically reduces both the number and functional capacities of NK cells [30]. However, various groups have also shown the presence of NKP and iNK cells in both mouse and human lymph nodes (LN), thymus, spleen, and liver [31-34] . The presence of NKP and iNK cells outside of the BM raises the question on whether or not the BM is only necessary in the initial steps of NK cell differentiation, which would then terminate elsewhere [1, 30]. Indeed, it is still not known if these NKP and iNK cells found outside of the BM proceed directly from the BM itself, or if they are distinct NK cell lineages.

Regardless of their origin, it has been postulated that NK cells which have egressed from the BM, recirculate and progress with their maturation in the periphery [30]. Non-iNK –NKP cells found in the periphery are usually subdivided into yet another three stages of maturation, which correlate with the gradual acquisition of effector functions. These stages have been defined by the acquisition of the expression of CD11b and CD27, in the following manner:

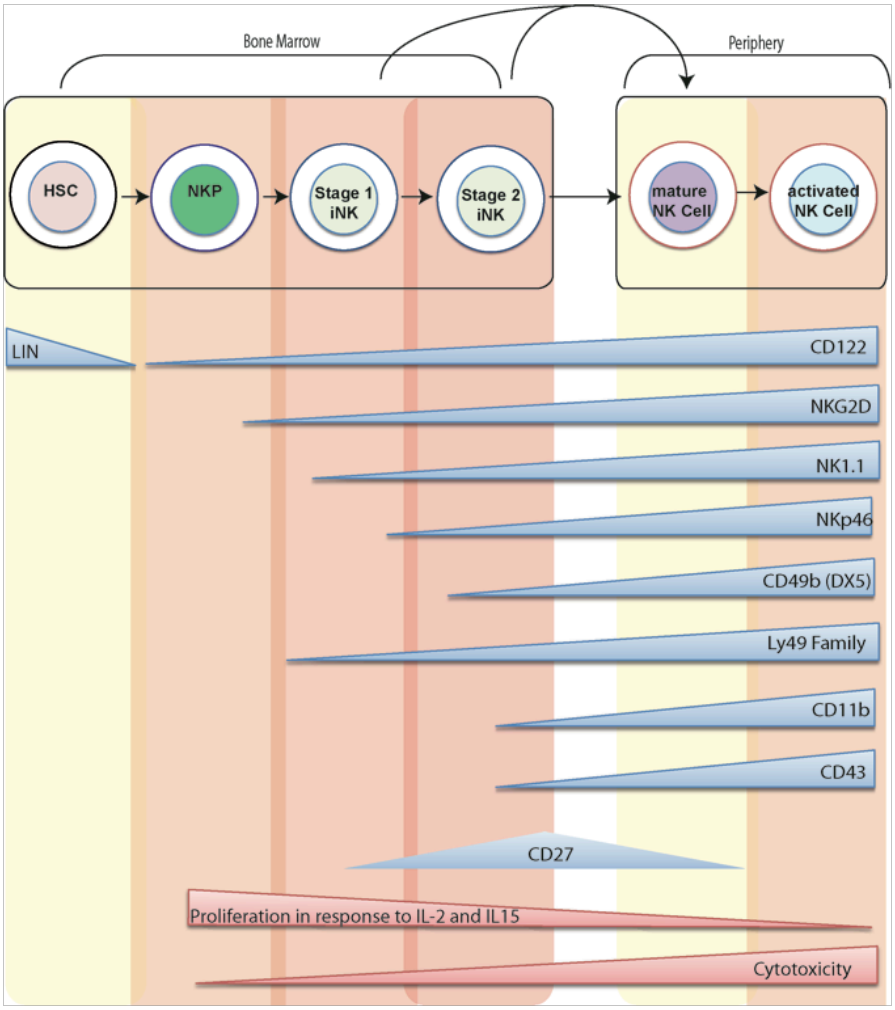

Figure I.1 – Phenotypic and functional acquisition of NK developmental markers

NK cells have been traditionally viewed as following a linear pathway from haematopoietic stem cells (HSCs) in the bone marrow which then differentiate and fully mature in the periphery. NK-cell precursors (NKPs) lack all typical NK-cell markers but are characterized by the expression of the IL-2 receptor β (CD122). These then give rise to NK cells via the sequential acquisition of developmental markers, such as the natural-killer group 2, member D receptor (NKG2D) and the NK-cell associated marker (NK1.1). It is thought that NK cell education via MHC-I class molecules occurs in the bone marrow, and that more-mature NK cells then leave and fully differentiate in peripheral organs. (modified Di Santo et. al, 2007, Nature Reviews)

$CD11b^{Low}CD27^{High}$ (the most immature), $CD11b^{High}CD27^{High}$ (intermediate maturation), and $CD11b^{High}CD27^{Low}$ (the most mature) [29, 35, 36]. **(Figure I.1)**.

The proportion of these three subsets varies greatly between tissues, and the main tissues which have been studied in detail, have mainly been BM, LN, spleen, liver, blood and lungs [37]. Within these tissues, immature $CD11b^{Low}CD27^{High}$ are predominantly found in LN and BM, while the more mature $CD11b^{High}CD27^{High}$ and $CD11b^{High}CD27^{Low}$ are usually found

in the liver spleen and lung [35, 38]. As is the case with the mechanisms behind iNK and NKP cells, it is still not known if $CD11b^{Low}CD27^{High}$ and/or $CD11b^{High}CD27^{High}$ arrive in these peripheral tissues directly from the BM and then mature directly in situ, or if the they directly derive from iNK cells **(Figure I.2)**. Nonetheless, it is known that splenic NK cells are capable of recirculating back into the BM, and could thus possibly be a part of the small, yet present mNK cell subsets which are found in BM **(Figure I.3)**.

The cytokines involved in the maturation of NK cells in vivo have still not been clearly identified, even though the importance of cytokines such as IL-12, IL-18, and IL-21 in vitro in generating unique mature NK cell phenotypes has already been established [39].

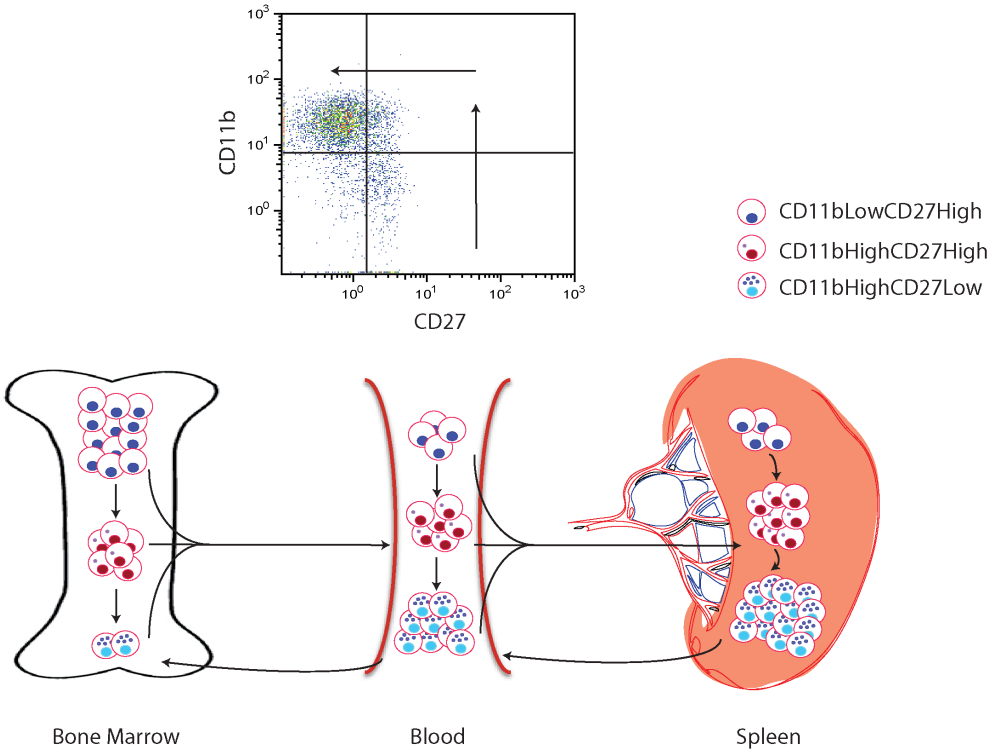

Figure I.2 – CD11b and CD27 NK cell subset circulation

Non-INK – NKP cells are usually subdivided into three stages of maturation which correspond to the acquisition of the CD11b and CD27 markers. NK cells mature from the $CD11b^{Low}CD27^{High}$ to the $CD11b^{High}CD27^{High}$ and then onto the $CD11b^{High}CD27^{Low}$ stage. It is thought that any of these NK cell subtypes can freely move from the bone marrow to any organ via the blood stream, and finish their maturation in situ. (modified Grégoire et.al, 2007, Immunological Reviews)

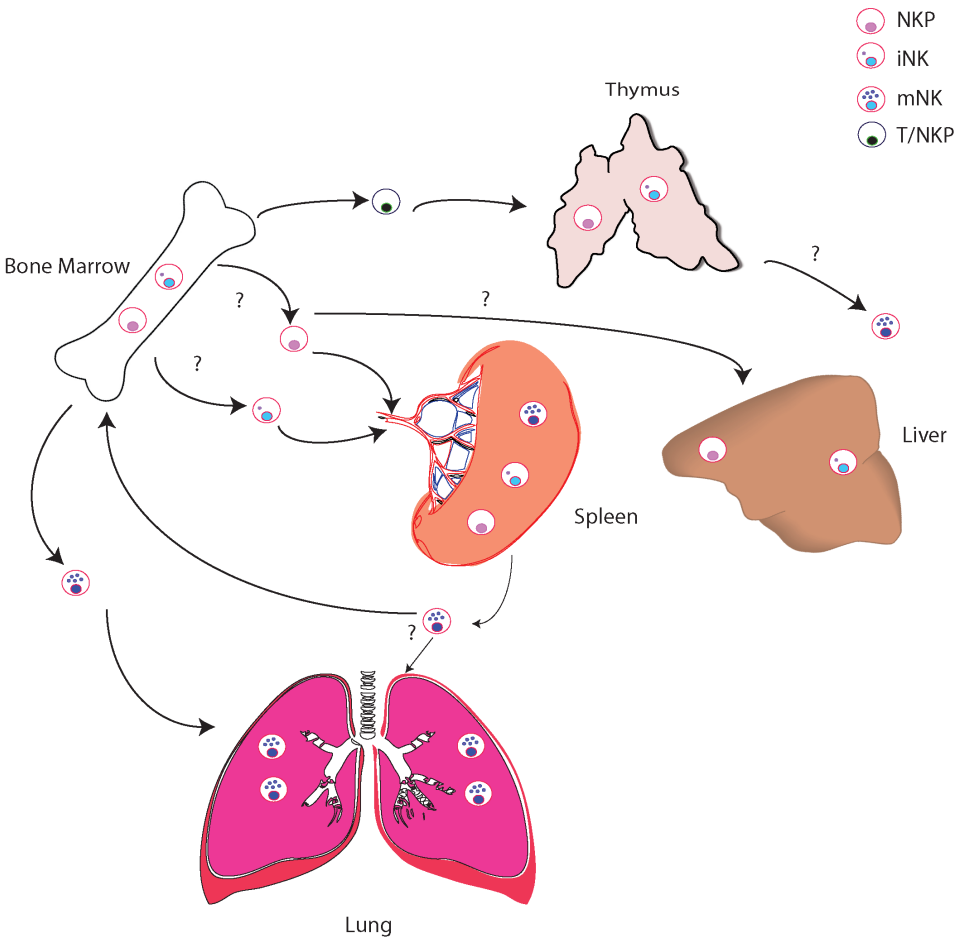

Figure I.3 – NK cell organ recirculation

Generation and recirculation of NKP, iNK and mature NK cell subtypes. NKP cells are thought to generate in the bone marrow, but both NKP, iNK and NK developmental intermediates have been found in other organs, such as liver and spleen. Certain NK cell specific subsets seem to reside in specific organs, such as mature $CD11b^{High}CD27^{Low}$ mature-NK cells in the lung and immature $CD11b^{Low}CD27^{High}$ NK cells in the spleen, but whether these intermediates actually complete their differentiation in situ or elsewhere is unknown. (modified Di Santo, 2006, Annu. Rev. Immunol.)

NK cell variations in organs

Interestingly, the frequency of NK cells in lymphocytes in mice is higher in the non-lymphoid organs such as the lung and liver compared to lymphoid organs, and the same tendency seems to occur in human non-lymhoid tissues [30, 40, 41]. The function and role of tissue-resident NK cells in different organs has been seen to vary greatly between organs. For example, liver-resident NK cells have been seen to play both a pro-inflammatory and anti-fibrotic role. Studies have reported how hepatic NK cells during viral infection have a higher cytotoxic phenotype, and produce higher levels of perforin and granzymes [42, 43]. On the other hand, liver

NK cells have also play an anti-fibrotic role, via their ability to deplete hepatic stellate cells, which are responsible for pro-fibrotic formation in the liver [44]. NK cells seem to exhibit the same janus-face also in mucosal tissues and skin. While lung-specific viral infections increase the flow of NK cells which produce large levels of TNF-α, NK cells in the gut seem to participate with other gut-associated lymphoid tissues in controlling a tolerogenic status to commensal flora [45, 46].

Part 2 – Natural Killer Cells in autoimmunity

Natural Killer Cells in Autoimmunity

Autoimmune disorders can be generically defined by an immune-mediated elimination or debilitation of endogenous cells and/or tissue. Autoimmune diseases are typically characterized via an inappropriate activation of cells of both the adaptive and innate immune system, which result in either specific or unspecific cell or organ damage. Autoimmune diseases can be characterized into various subcategories; 1) an unrecognized ongoing infection in the target organ 2) a cross-reaction with an environmental antigen (molecular mimicry) 3) bad cell death, with subsequent necrotic and not apoptotic cell death 4) the autoimmune response is a result, not a cause, of the disease (immune markers) [47]. Notwithstanding, autoimmune diseases seem to be initiated in a step-wise manner, involving the release of self-antigen from target organs which in turn prime secondary lymphoid organs which ultimately leads to tissue destruction [48] . NK cells are capable of partaking in any of the aforementioned steps, seeing as how they can mediate initial target damage via the lysis of cells expressing "stress" self-antigens and also by being active immunomodulators and effector cells in target organs [48-50].

NK cells, the activating NGK2D receptor, and NKG2D ligands have been implicated in various autoimmune diseases such as type 1 diabetes (T1D), rheumatoid arthritis (RA), and Crohn's disease [51-56]. Just as NK cells seem to have different functions depending on their peripheral localization, so do NK cells seem to behave differently in the various autoimmune diseases. As Perricone et. al suggested, there seems to be a great variability within and between autoimmune diseases into when NK cells act as either shields against autoimmunity, or swords which enhance it **(Figure I.4)** [49].

In multiple sclerosis (MS), NK cells seem to behave as "shields", since patients in remission from MS seem to exhibit high percentages of Th2 cytokine secreting NK cells (such as IL-5 and IL-13) [57, 58]. In rheumatoid arthritis, NK cells are greatly present in the inflamed joints, and recent advances have shown how synovium-infiltrating NK cells from patients produced more IFN-γ and were phenotypically more active compared to peripheral blood NK cells from

the same patients [59, 60]. The possible role that NK cells might have in systemic lupus erythematosus (SLE), has not however been discussed at length.

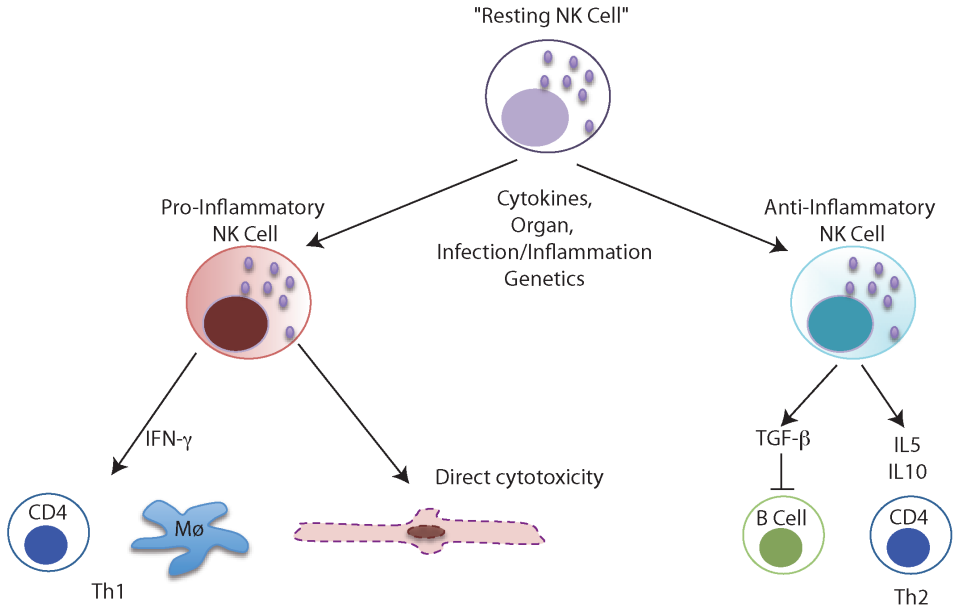

Figure I.4 – Pro- and anti-inflammatory NK cells

Various factors are capable of modulating an NK cells capacity to either regulate or down-regulate the immune response. These factors, which can encompass such distinct stimuli such as they cytokine mileu, organs, the inflammatory/infectious status, or genetic background. Pro-inflammatory NK cells can either release cytokines such as IFN-γ which activate Th1 type T cells and macrophages, or can directly lyse target tissue cells via the release of perforin and granzyme. NK cells can also play an anti-inflammatory role in by producing Th2 type cytokines such as IL-5, Il-10 and TGF-β. (modified Johannson et. al, 2005, TRENDS in Immunology)

Systemic Lupus Erythematosus

SLE is a chronic autoimmune disease that can affect various organs, including the heart, lungs, blood, kidneys and nervous system [61, 62]. The etiology of this disease remains unknown, and various studies postulate roles for genetic and/or environmental factors in its development [61]. The hallmarks of SLE are auto-antibody production to a broad range of self-antigens, immune complex formation, and self-reactive T and B cells able to produce these auto-antibodies [63-65]. Immune complex formation in kidney glomeruli is presumed to be an initiator of lupus nephritis, one of the main causes of mortality and morbidity in human SLE and in murine SLE models [66]. Once these complexes appear, interstitial infiltrates of macrophages, T and B cells form, in turn amplifying the local inflammatory response [67, 68]. This accumulation of cell infiltrates correlates with severity of the glomerular lesions, leading in turn to glomerulonephritis and eventual renal failure [69, 70].

Natural Killer Cells in SLE

The role of NK and of NKG2D+ cells in the development of SLE pathogenesis remains unclear and is widely debated. Recent reports from several groups show a significantly lower proportion and total numbers of NK cells in SLE patient blood compared to controls, especially in patients with lupus nephritis [71-73]. This reduction in the total numbers of NK cells in the peripheral blood seems to be a common hallmark in various autoimmune diseases, such as MS, RA, T1D, and Sjögren Syndrome, even though its significance is not understood [49, 74, 75]. There also is evidence of reduced cytotoxicity and impaired differentiation in NK cells from SLE patients and from mouse SLE models such as MRL/MpJ^{lpr} [71, 76, 77]. Other studies showed a larger proportion of CD4+NKG2D+ cells in the peripheral blood mononuclear cells (PBMC) of SLE patients, although an inverse correlation is reported between CD4+NKG2D+ cells and disease activity in juvenile-onset SLE patients [52, 78]. Higher levels of the NKG2D MICA ligand were also found in juvenile-onset SLE patient serum, suggesting NKG2D ligand upregulation in SLE pathogenesis [52]. There are nonetheless few data regarding the role of NKG2D and its ligands in SLE pathogenesis and etiology, or on the functional characterization of NK cells in a SLE environment, specifically in target organs of this disease.

Part 3 – The NKG2D receptor and its signaling pathway

NKG2D activating receptor

One of the main NK cell activating receptors is the NKG2D activating receptor which is expressed on most NK, NKT, activated CD8 T cells, and γδ T cells [12, 17, 51]. NKG2D is a highly conserved, non-ITAM (immunoreceptor tyrosine-based activation motif), C-type lectin-like, type II transmembrane glycoprotein which is encoded by a single gene [79]. NKG2D in mice exists both in a long (NKG2D-L) and short (NKG2D-S) form which are generated via alternative RNA splicing [80]. The NKG2D-L protein associates with DAP10, a trans-membrane signaling subunit, while the NKG2D-S can pair either with DAP10 or DAP12, another trans-membrane subunit. The human trans-membrane unit of NKG2D can only associate with DAP10 and not DAP12 **(Figure I.5)**. While it has been seen that the NKG2D-DAP10 signaling pathway plays a crucial role in NKG2D mediated cytotoxicity, the role DAP10 and DAP12 have in NKG2D mediated cytokine production is less clear [17]. Indeed, DAP10 deficient mice which only express NKG2D-DAP12 complexes, are capable of cytokine secretion and cytotoxicity when NKG2D is crosslinked, while in contrast DAP12 deficient mice which only express

NKG2D-DAP10 complexes, cytotoxicity but not cytokine production is induced [17, 80-82].

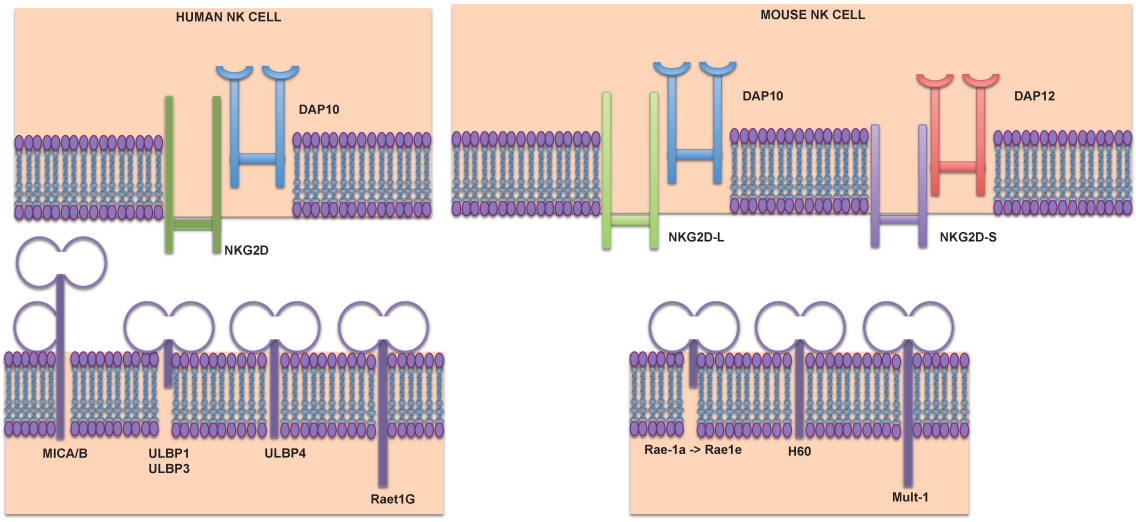

Figure I.5 – The NKG2D receptor and its ligands

Natural-killer group 2, member D (NKG2D) is constitutively expressed by NK cells in both humans and mice. In humans, NKG2D associates exclusively with the adaptor protein DAP10, assembling a hexameric complex. In mice, alternative splicing of NKG2D results in both a long-form (NKG2D-L) which can associate with DAP10, or a short-form (NKG2D-S) which can associate either with DAP10 or DAP12. In mice NKG2D mediated signaling depends on if the NKG2D receptor is associated either with DAP10 or DAP12. There are seven NKG2D ligands expressed in humans and mice. All of the ligands have MHC-I related domains. NKG2D ligands are expressed at the cell surface in response to cellular stress which can be caused by factors such as DNA damage and/or viral infection.

DAP10 signaling

DAP10 mediates NKG2D signaling via a 21 amino acid signaling motif (YINM), which is capable of binding either the p85 subunit of phosphatidylinositol-3-OH kinase (PI3K) or growth factor receptor bound protein 2 (GrB2) when phosphorylated[17]. These two signaling pathways haven been seen to play different, yet collaborative roles. Abrogating the binding capacities of either the p85 subunit or inhibiting Grb2 signaling via the disruption of the Vav-1 protein induces both impaired NKG2D mediated cytotoxicity and defective actin cytoskeleton reorganization after target-cell contact [83-85] **(Figure I.6)**.

DAP10 has also been seen to play a role in IL-15 mediated signaling. It has been shown that transgenic NK cells lacking the DAP10 signaling subunit have defective IL-15 responsiveness [86]. This defect in IL-15 responsiveness was due to the coupling of DAP10 and IL-15 receptor signaling in NK cells. Indeed, not only does DAP10 interact with the IL-15R complex, but its absence completely abolishes STAT5 mediated signaling via the IL-15 receptor in NK

cells [86]. STAT5 is a member of the STAT family of proteins, which signal via the JAK/STAT pathway. STAT5 is central to the modulation and signaling of the IL-15 receptor after stimulation with IL-15 [87].

Role of PI3K in Natural Killer Cells and NKG2D signaling

Class I PI3K enzymes are formed by a p110 catalytic subunit and a regulatory subunit; the p110 subunit catalyzes formation of phosphatidylinositol P2 and phosphatidylinositol P3 after receptor stimulation. Class Ia are heteorodimeric molecules composed of a p110 catalytic subunit (α, β, or δ) and a p85-like regulatory β subunits (p50α, p55α, p85α, p85β). The expression of p85α and p85β have been seen to be ubiquitous, unlike the p50 and p55α subunits whose expression is restricted to certain tissues [88].

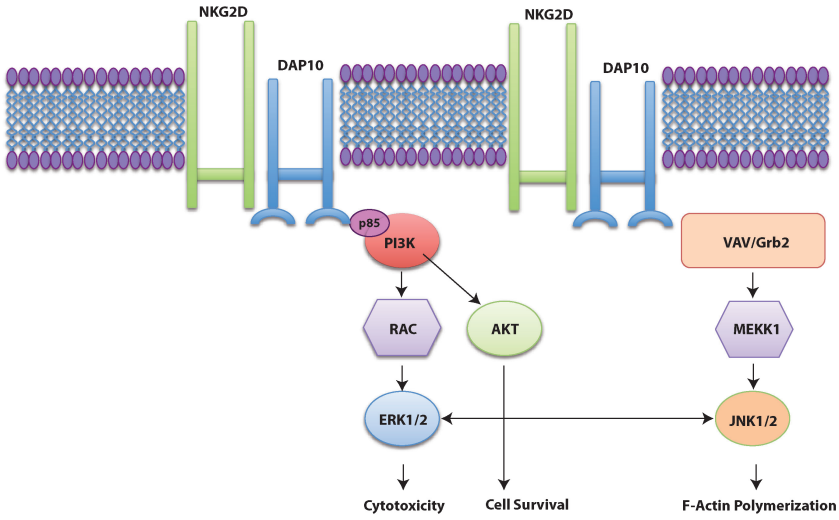

Figure I.6 – NKG2D-DAP10 signaling pathway in NK cells

NKG2D activation leads to the recruitment of the p85 regulatory subunit of PI3K and/or to the recruitment of the Grb2/VAV complex to the phosphorylated YINM motif in the cytoplasmic domain of DAP10. These events are then capable of various downstream kinases, such as AKT, ERK, and JNK which initiate and control events such as F-actin polymerization, NK cell survival, and NK cell mediated cytotoxicity.

Various studies have already shown the importance of the PI3K pathway in NK cells. Studies using both knockout and knockin mouse models have already shown the role of the p110γ and p110δ PI3K subunits in NK cell maturation, development and cytokine generation [89-91]. However little is known about the potential role that the p85β subunit has in NK cells, even though the importance of the p85 binding site, as previously stated, has already been reported. The abrogation of the p85 binding site abrogates DAP10 mediated cytotoxicity in human natural killer cells [92]. It still remains to be ascertained though if the p85 binding site

binds either the p85α, p85β or both subunits. p85β has already been reported to bind with greater affinity to CD28 rather than p85α in murine T cells, and it has also been reported to be implicated in the internalization of the KIT receptor in mast cells, thus prompting the question if whether or not it might also have a role in mediating NKG2D receptor signaling [93, 94].

Objectives

The principal objectives that were established at the beginning of this experimental thesis were the following:

1) Phenotypically and functionally characterize NK cells in murine SLE-like models both in target and non-target organs of the disease.

2) Ascertain if NKG2D ligands are expressed in the kidneys of healthy and/or diseased murine SLE-like models and if they correlate with glomerulonephritic onset.

3) Characterize the NK cell population in p85β knockout mice and analyze the possible functions of the p85β subunit of PI3K in NKG2D signaling

Materials and Methods

Nullius addictus iurare in verba magistri

Horace

It is the rule which says that the other rules of scientific procedure must be designed in such a way that they do not protect any statement in science against falsification.

Karl Popper

Mice

Female C57BL/6J, MRL/MpJ, and MRL/MpJ^{lpr} (Jackson Laboratories) mice were maintained in the CNB animal facility in pathogen-free conditions, and were sacrificed at 9, 15, or 52 weeks of age. FVB-121, FVB-187 and their respective wildtype littermates were generated as previously described [95]. They were bred and maintained by facilities pertaining to either Cancer Research UK or the Peter Gorer Department of Immunobiology (King's College). All animal studies performed in the UK were in compliance with the UK Home Office.

p85β deficient mice (p85β$^{-/-}$) were generated via the disruption of the first exon of the Pik3r2 gene by homologous recombination, as has been previously described [96]. They were maintained in the CNB animal facility in pathogen-free conditions.

All animal studies were approved by the CNB Ethics Committee for Animal Experimentation (Ref. 11022 and Ref. 12033) in compliance with national and European Union legislation.

Cell lines

CHO cells were cultured in F-12 medium with 10% FBS. YAC-1 cells (American Type Culture Collection) were cultured in RPMI 1640 with 10% FBS. RMA/S cells (a kind gift of Dr. B. Chambers, Karolinska Institute, Stockholm, Sweden) were cultured in RPMI 1640 with 10% FBS and 2 mM L-glutamine.S2 cells (a king gift of Dr. E Long, National Institute of Allergy and Infectious Diseases, Maryland, USA) were cultured in Schneider`s Drosophila Medium with L-glutamine (Lonza) with 10% FBS. S2 cells were grown in a 27°C incubator. Stably transfected S2 cells were cultured in 10% FBS and 0.5 mg/ml of G418 (Genycell Biotech).

Generation of stable S2 Cell lines

The plasmid for the constitutive expression of MULT-1 and mICAM1 in S2 cells was the pAc5.1-V5-His plasmid (Invitrogen) whose expression is driven by the Drosophila Actin 5 promoter. The pAc5.1–V5–His-mICAM1 (with the murine ICAM1 cloned insert) plasmid was a kind gift from Dr. E. Long. The pAc5.1–V5–His-Mult1 plasmid (with the murine MULT-1 cloned insert) was generated with the following strategy. The pcr4-MULT-1 plasmid (IRCKp5014H065Q; imaGenes) was digested with the XcmI enzyme (New England Bioscience) and the ends blunted using Klenow enzyme (New England Bioscience). The plasmid was then digested with XbaI (New England Bioscience) and the MULT-1 insert was extracted. The pAc5.1-V5-His plasmid was digested with XcmI, and the ends blunted with Klenow enzyme. The pAc5.1-V5-His plasmid was then digested with Xba-1, and the MULT-1 insert from the

pcr4-MULT-1 plasmid subcloned and ligated into the pAc5.1-V5-His plasmid using T4 DNA Ligase (New England Bioscience). Resulting clones were confirmed by sequencing using the AC5 Forward (5' ACACAAAGCCGCTCCATCAG 3') and the BGH Reverse (5' TAGAAGGCACAGTCGAGG 3') primers. Stable transfectants of S2-Mult1, S2-Mult1/mICAM1, S2-mICAM1, were generated via transfection of the pAc5.1-V5-His-mICAM1 and pAc5.1-V5-His-Mult1 plasmids with the Calcium Phosphate Transfection Kit (Invitrogen). Briefly, S2 cells ($1*10^6$) were plated in a well of a 6-well plate O/N, and the day of transfection, per each transfection, 19 µg of DNA and 1 µg of pNeofly (selection plasmid for neomycin) [97] were added to a 2 M CaCl2 mixture and then to HBS. This transfection mix was then added dropwise to the S2 cells, and the cell medium was changed after 24 hours. G418 as a selection marker for transfection was added after 48 hours, and transfection efficiency was assayed via flow cytometry **(Figure M.1)**. In order to equalize

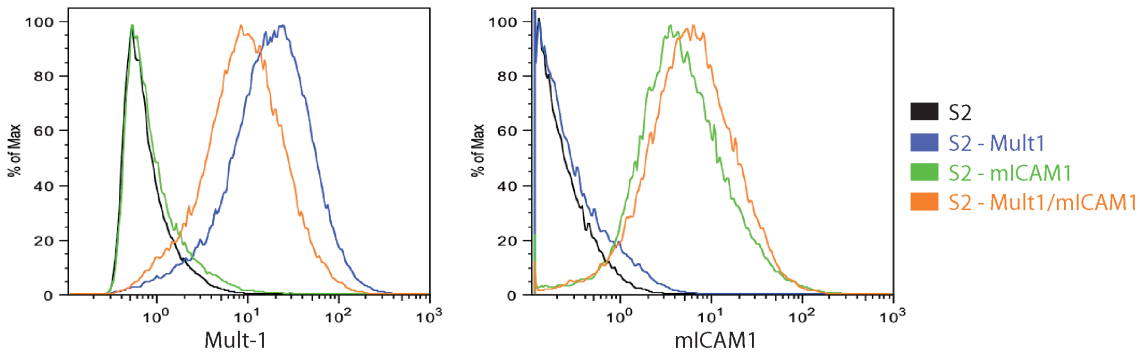

Figure M.1 – Expression of murine ligands on transfected S2 cells

Schenider Drosophila 2 cells (S2) were stably transfected with plasmids containg murine ICAM1 (mICAM1) and/or the MULT-1 NKG2D ligand. S2 cells were maintained in G418 for selection of positively transfected cells. S2 cells were stained with fluorochrome-conjugated anti-ICAM1 or anti-MULT-1 mAb and analyzed via flow cytometry.

levels of expression of MULT-1 and mICAM1 between single and double positive S2 cells, transfected S2 cells were sorted based on the expression levels of mICAM1 and MULT-1. S2-mICAM1, S2-MULT-1, and S2-MULT-1/mICAM1 cells were stained for either mICAM and/or MULT-1 on a FACS Aria Ilu Cell sorter (Beckton Dickinson) and levels of expression were checked after 2-3 weeks post sorting.

Cell isolation

Single-cell suspensions of mouse spleen and BM (from tibiae and femur) were obtained by passing suspensions through a 40 µm nylon cell strainer, followed by lysis of erythrocytes

using an erythrocyte lysing buffer for 5 minutes at room temperature. To obtain kidney lymphocytes, decapsulated kidneys were minced, digested with 10 μg/ml collagenase D (Roche; 37°C, 10 min), passed through a 40 μm nylon cell strainer and washed 3 times with 50 ml PBS with 3% FBS and 2 mM EDTA. Cells were resuspended in RPMI with 3% FBS, gently overlaid on Ficoll (GE Healthcare), and centrifuged (700 x g, 20°C, 20 min). Kidney lymphocytes were isolated from the interface and washed twice with PBS. Kidney lymphocytes were counted using a hemocytometer, and the absolute number of NK, CD3, CD4, and CD8 cells in kidneys was calculated by multiplying total kidney lymphocytes by the percentage of positive cells as determined by flow cytometry.

Cell staining and flow cytometry

Antibodies to the following antigens were used for staining: B220 (RA3-6B2; Beckman Coulter), CD4 (GK1.5; eBioscience), CD8 (53-6.7; eBioscience), CD54 (YN1/1.7.4; eBioscience), MULT-1 (5D10; eBioscience), Ly-6G/Ly-6C (RB6-8C5; Biolegend), NKp46 (29A1.4; Biolegend), CD2 (12-15; Southern Biotechnology), MULT-1 (1D6), CD3 (145-2C11; eBioscience), NKG2D (A10; eBioscience); CD11a (2D7), CD11b (3A33), CD27 (LG.3A10), CD43 (S7), CD49b (DX5), CD51(RMV-7), CD107a (1D4B), CD122 (5H4), CD45 (I3/2.3), IFNγ (XMG1.2), TER119 (TER119), Ly49A+Ly49D (12A8), Ly49C+Ly49I (14B11), Ly49D (4E5), Ly49F (HBF-719), Ly49G (4D11), pSTAT5 (pY694) were all purchased from BD Biosciences. Dead cells were distinguished using the Live/Dead Fixable Dead Cell Stain (L34955, Invitrogen).

Natural killer precursor cells were gated on the $CD122^{+}Lin^{-}$ (Lineage) gate, in which the Lin panel included the following antibodies: anti-B220, -CD3, CD11b, Ly-6G/Ly-6C, and TER119. Stained cells were analyzed in a Gallios (Beckman Coulter) flow cytometer and data analyzed with Kaluza v. 1.0 (Beckman Coulter) and FlowJo v. 8.0 (Treestar Inc.) software.

NK cell isolation

Single-cell suspensions from spleen and BM were passed through nylon wool columns to deplete adherent populations consisting of B cells and macrophages [98]. Nylon wool non-adherent cells were then purified with the CD49b microbead or the anti-NKp46 microbead kits (Miltenyi Biotech). Cells were cultured with 20 nM murine IL-15 or murine IL-2 (1000 U/ml) (both from Peprotech) in RPMI with 10% FBS, penicillin, streptomycin, L glutamine, sodium pyruvate, non-essential amino acids, and beta-mercaptoethanol.

Conjugation assays

Six-day IL-15-cultured or IL-2-cultured NK cells were tested for purity and used in conjugation assays. For conjugation assays, spleen and BM NK cells were stained with PH26 Red Fluorescent Cell Linker, and YAC-1 or S2-mICAM1 cells with PKH67 Green Fluorescent Cell Linker (both from Sigma-Aldrich). NK and target cells were coincubated at a 1:2 ratio (37°C, various times), fixed in 1% paraformaldehyde, and analyzed by FACS. Events positive for red and green fluorescence were considered conjugates, and the percentage of conjugation was calculated as (conjugated NK cells/total NK cells) × 100.

CD107a degranulation Assays

Six-day IL-15-cultured or IL-2-cultured NK cells were tested for purity and used in degranulation assays. 2 x 105 NK cells were incubated at a 1:2 ratio with different target cells (37°C, 4 h) with 10 µg/ml monensin (Sigma) and 5 µl CD107a-FITC-conjugated antibody or an isotype control. Cells were washed and stained with anti-NKp46 and -CD3 antibodies. The percentages of CD107a-positive $NKp46^{+}CD3^{-}$ cells were analyzed by flow cytometry

Natural Killer Cytotoxicity FACS Assay

Six-day IL-2-cultured NK cells were tested for purity and used in cytotoxicity assays. For cytotoxicity assays, target cells were stained with PKH67 Green Fluorescent Cell Linker (Sigma-Aldrich). NK and target cells were coincubated at various ratios (1:1, 1:3, 1:5, 1:10, 1:20) in 200 µl total volume for 4 hours at 37°C. Each condition was done in duplicate. The reaction was stopped by adding 400ul of ice-cold PBS-staining and placing the tubes on ice. 15 ul of PI was added to each cell before passing the tube on the flow cytometer. Target cells were gated for bright positivity on the FL1 gate. The percentage of specific lysis of each target cell in each condition was calculated by applying the following formula: (100x(%PI positive cells - % Spontaneous death)/(100 - % Spontaneous Death)). A positive control for cell death consisting of only target cells incubated with 3% H_20_2 was included in every experiment.

Intracellular IFN-γ staining

Freshly isolated kidney lymphocytes in RPMI 1640 supplemented with 10% FBS were cultured with PMA (P8139; Sigma; 25 ng/ml), ionomycin (I0634; Sigma; 1 µg/ ml), and brefeldin A (420601; Biolegend; 1X) (4 h, 37°C, 5% CO2). Cells were harvested after culture, stained

with antibodies to surface antigens (30 min, 4°C), fixed with 4% paraformadehyde, permeabilized with PBS 0.5% saponin, and stained for IFN-γ (diluted in permabilization buffer, 30 min, room temperature (RT)). Cells were washed once with permeabilization buffer and analyzed by flow cytometry. Control isotypes were included in all experiments for intracellular cytokine detection.

pSTAT5 staining

Freshly isolated kidney lymphocytes and single cell suspensions from spleen and BM were washed and incubated (15 min) in RPMI 1640 alone or with 20 nM recombinant murine IL-15; the reaction was terminated by adding the same amount of BD Cytofix buffer (554655; BD Biosciences). Cell suspensions were washed, permeabilized with BD PhosphFlow Perm Buffer III (558050; BD Biosciences), and stained for surface antigens. Cells were then washed, and stained with pSTAT5 (pY694) diluted 1:1 in PBS.

Total Organ Elisa

Mice were sacrificed, and the kidneys of the mice of each group were exiced, weighed, and finely minced. Kidneys were then plated in 2 ml of RPMI with 10% FBS, penicillin, streptomycin, L glutamine, sodium pyruvate, non-essential amino acids, and beta-mercaptoethanol at 37°C for 24 hours. Supernatants were collected, and spun down three times at 300 x RCF in order to remove cellular debris. The supernatants were then tested in triplicate for IL-15 (eBioscience Mouse IL-15/IL-15R Complex ELISA Ready-SET-Go!), CXC3CL1 (RnD Systems Mouse CX3CL1/Fractalkine DuoSet), and IL-12 (BD OptEIA Mouse IL-12 (p70) ELISA Set). The final concentration was then normalized to the total weight of each organ.

Immunohistochemistry and immunofluorescence antibodies and reagents

We used rat anti-mouse Ly49G2 (4D11, 1/100; eBioscience), goat anti-mouse NKp46 (AF2225, 1:100; R&D Biosytems), rat anti-mouse CD8 (YTS169.4, 1/100; abcam), rat anti-mouse CD4 (RM4-4; 1:100; Biolegend), goat anti-mouse Rae1γ (AF1136, 1:100; R&D Biosystems), rabbit anti-mouse synaptopodin (1:200; Synaptic Systems), rat anti-mouse Mult1 (1D6, 1:50), goat anti-mouse H60 (C-20; Santa Cruz Biotechnologies), rat anti-mouse H60 (205326; R&D Biosystems), goat anti-human MICA (BAF1300, 15μg/ml; R&D Biosystems), rabbit anti-human ULBP1 (1:30; Novus Biologicals), donkey anti-goat IgG Alexa488, donkey anti-rabbit IgG Alexa594, mouse anti-rat Cy3 (all three from Jackson ImmunoResearch), and DAPI. Rat

anti-mouse Mult1 was a kind gift from Dr. S. Joncic (Univ. Rijeka, Rijeka, Croatia) [99], aged NZBxNZW(F1) OCT-embedded kidney tissue sections were a kind gift from Dr. S. Izui (Univ. Geneva, Geneva, Switzerland), and three-month-old female BALB/c kidney tissue sections were a kind gift from Dr. M. Zonca (CNB, Madrid, Spain).

Immunohistochemistry protocol

Spleens and kidneys were extracted and snap-frozen in tissue-freezing medium (Jung). Sections (10 μm) were acetone-fixed (4°C, 10 min), endogenous peroxidase was blocked (K4009; Dako; 7 min) and slides incubated with primary antibody (2 h, RT or overnight, 4°C), followed by rabbit EnVision+ System HRP reagent (Dako; 30 min), rat or goat Histofine Simple Stain kits (Nichirei Biosciences; 30 min). Frozen sections were stained with AEC+ substrate chromogen solution (Dako; 15-20 min) and hematoxylin-counterstained (Sigma-Aldrich; 3 min). All mAb and antisera were diluted in PBS with 10% FBS and 2.5% BSA, and used at concentrations determined to yield optimal staining. HRP-conjugated polymer-stained sections were used as negative controls.

Formalin fixed kidney sections from six patients with confirmed active Stage III-IV Lupus Nephritis were stained for ULBP1 and MICA expression. Sections were deparaffinized in xylene and rehydrated in EtOH. Heat Induced Antigen Retrieval was performed using Tris-EDTA (ph 9.0) for 20 minutes in a water steamer. Sections were washed, endogenous peroxidase blocked, and primary antibodies for MICA and ULBP1 left overnight at 4°C. Sections were washed, and staining was followed by rabbit EnVision+ System HRP reagent (DAKO; 30 min) or Vectastain Elite ABC Kit (Vector Laboratories; 30 min). Sections were stained with AEC+ substrate chromogen solution (Dako; 15-20 min) and hematoxylin-counterstained (Sigma-Aldrich; 3 min). HRP-conjugated polymer-stained sections were used as negative controls.

Quantification of Rae-1 staining intensity in glomeruli

Chromogen deposition was measured by quantitative immunohistochemistry using an established method [100]. In brief, images of glomeruli (100x magnification) from various mice were acquired in a Leica microscope (vertical Leitz DM RB) with an adapted Olympus (DP70) camera; image files were saved in a tagged-image file format. The amount of chromogen per pixel was determined by selecting the glomeruli in a 200 x 200 pixel region and subtracting the mathematical energy (EM) of the control slide (not exposed to primary antibody) from that of a homologous glomerulus on the experimental slide (exposed to Rae-1 antibody). Chromogen quantity (EM) is expressed as energy units per pixels (eu/pix). We analyzed 25 glomeruli from

each mouse in every group. Data are represented as mean ± SD. Statistical analysis was performed using a two-tailed Student's t-test.

Immunofluorescence and confocal analysis

Kidney sections (10 μm) were acetone-fixed (4°C, 10 min), blocked with serum (2 h), incubated with primary antibodies (RT, 2 h), followed by secondary antibodies (RT, 45 min). Cells were washed in TBS, incubated with DAPI (15 min), and mounted with Fluoromount G (Southern Biotech). Confocal analysis was performed on a Leica SP5 confocal microscope. Entire-tissue section pictures were analyzed via immunofluorescence using a Leica DMI 6000B inverted microscope and the Leica Application Suite Microscope Software to create a full processed image. All samples include appropriate antibody staining controls.

Quantification of stained area and statistical analysis

Stained areas were quantified using ImageJ (100x). For quantification of the total stained areas of kidneys, 50 representative sections were analyzed from 5 mice from each group. To quantify the percentage of glomeruli with $NKp46^+$, $CD4^+$, or $CD8^+$ infiltration, 25 random field sections were analyzed for 5 mice from each group. In each section, the total number of glomeruli was counted, then divided by the number of glomeruli which showed at least one positive-staining AEC cell inside the glomeruli. Data are represented as mean ± SD. Statistical analysis was performed using a two-tailed Student's t-test. Data were considered significantly different at a value of $p < 0.01$ and highly significant at $p < 0.001$.

NK cell BrdU proliferation assay

Splenic NK cells were cultured for 6 days, harvested, and seeded into a 6 well plate ($2*10^6$ cells/well) in complete RPMI medium for 2 hours. BrdU was then added to a final concentration of 10 μM, and cells were incubated for 4 hours. Cells were then surface stained for NKp46, CD3 on ice for 30 minutes, washed, and then fixed with 1% paraformaldehyde-0,01% Tween-20 in PBS while vortexing. Cells were incubated at RT for 30 minutes and then for 30 minutes on ice. Cells were washed, and resuspended in a solution containing 50 Kunitz DnaseI/ml (0,15M NaCL, 3,2 mM MgCl, 10 μM Hcl). Cells were incubated for 10 inutes at RT, washed, and stained with anti-BrdU-FITC (BD Biosciences).

IFN-γ Elisa

Wells of 96 well-flat bottom plates were coated overnight at 4°C with 10 μg/ml of either anti-NKG2D (A10) or anti-NK1.1 (PK136) mAbs in PBS. Plates coated only with PBS served as controls. Wells were washed three times with PBS, and $2*10^5$ IL-2-activated splenic $NKp46^+CD3^-$ cells were plated per well for 4 hours. After 4 hours the supernatants were collected, and tested in triplicate for IFN-g via a commercially available ELISA kit (BD OptEIA Mouse IFN-γ ELISA Set).

NKG2D Downregulation Assay

NKG2D downregulation assays were performed with six day IL-2-activated spleen derived NK cells from $p85\beta^{-/-}$ and WT mice. $1*10^6$ cells per condition were stimulated on ice, in ice-cold PBS with 10 ug/ml NKG2D A10 activating antibody for 15 minutes. The reaction was stopped by adding 200 ul of ice-cold PBS and cells were spun down for 5 min x 300 RCF at 4°C. Cells were then incubated with an anti-hamster IgG2b antibody (G94-56) at 37°C in a warm water bath at various time points. Reaction was stopped by adding double the volume of PFA 4%. Cells were then stained for 30 minutes at 4°C with anti-CD3, -NKp46, -NKG2D (CX5 clone) antibodies. Cells were then analyzed via flow cytometry. The percent of downregulation in each condition was calculated relative to the MFI of NKG2D staining of cells which had been stopped at time point 0.

qRT-PCR analysis of gene expression

RNA was extracted from six day IL-2 activated spleen derived NK cells from C57BL/6 mice. RNA was extracted from NK cells using the Purelink RNA mini kit (Ambion Lifesciences). cDNA was obtained using total RNA using the High Capacity cDNA Reverse transcription kit (Applied Biosciences). Oligonucleotide primers used were of murine p85α (Forward: CATCTCCAAGTCCACTGACG Reverse: GAATGTTCGACTCTATACAGAACACAA), p85β (Forward: GCGGTAGATGACACAGTGCTT Reverse: GGGCTGTTACGCATGCTC), and β-actin (Forward: GGCTCCTAGCACCATGAAGA Reverse: CCACCGATCCACACAGAGTA). PCR reactions contained Power SYBR Green RT MasterMix (Applied Biosciences) and amplification was performed in an ABI PRISM 7900HT (Applied Biosystems). Amplification was performed at 95ºC, 10 min; 40 cycles, 95ºC, 15 sec; 60ºC, 20 sec; 72ºC, 30 sec. mRNA levels were quantified using the $2^{-\Delta Ct}$ method and normalized to β actin. Results were analyzed using SDS v2.2.2.

Results

Just because an idea is true doesn’t mean it can be proved. And just because an idea can be proved doesn’t mean it’s true.”

Jonah Lehrer

You always admire what you really don’t understand.

Blaise Pascal

Part 1 NK cell characterization in the MRL mouse strain

1.1 Natural killer cell cellularity in the MRL mouse strain

The MRL mouse strain spontaneously generates a lupus-like phenotype, one of the main effects of which is severe glomerulonephritis. MRL/MpJ (MPJ) mice develop this phenotype spontaneously at ~12 months of age, whereas MRL/MpJ^{lpr} (LPR) mice, which have the same genetic background but which generated a spontaneous homozygous mutation in the Fas allele, develop similar but more severe symptoms by ~3-4 months of age [101]. However, while the LPR mouse has been extensively used as a SLE-like model, scarce data is found in the literature using the MPJ mouse as a model for the study of SLE. The limited phenotypic characterization of NK cells in a SLE environment prompted us to characterize the NK cell population in BM (BM) and spleen from healthy and diseased MPJ and LPR mice; this would allow us to assess whether defects reported in the periphery (spleen) are also found in BM, where NK cells develop. We analyzed total splenocyte and BM cell numbers in predisease LPR (9-week-old), diseased LPR (3- to 4-month-old), healthy MPJ (3- to 4-month-old) and diseased MPJ (1-year-old) mice. Nine-week-old LPR mice were used as a pre-disease model due to absence of skin lesions, inflammation or cell proliferation in kidneys. One-year-old MPJ mice were sacrificed as soon as severe skin lesions appeared. As predicted, significantly larger numbers of splenocytes were seen in diseased and pre-diseased LPR mice (splenomegalia is a common feature due to the LPR mutation) compared to healthy and diseased MPJ mice **(Figure R.1A)**. We found no difference in total splenocyte number between healthy and diseased MPJ mice, and no differences in total BM cellularity between groups **(Figure R.1A,B).**

To determine the proportion of NK cells in spleen and BM, we gated the NKp46^{+}CD3^{-} cell population. NKp46 belongs to the natural cytotoxicity receptor family and is expressed specifically on NK cells and a subset of NK-like T cells [102, 103]. Furthermore, its expression does not appear to be strain-dependent, at difference from the NK1.1 protein which is not expressed in the MRL background [102]. We observed a significant reduction in the percentage of spleen NKp46^{+}CD3^{-} cells in diseased LPR mice compared to prediseased LPR and all MPJ mice; there was also a difference between diseased and healthy MPJ mice **(Figure R.1E)**. Diseased MPJ mice show significantly lower total spleen NKp46^{+}CD3^{-} cell numbers than any of the other groups **(Figure R.1F)**; no differences were found in total NKp46^{+}CD3^{-} cell numbers in the other groups. There were no differences in total number or percentage of NKp46^{+}CD3^{-} cells in BM **(Figure R.1G,H)**.

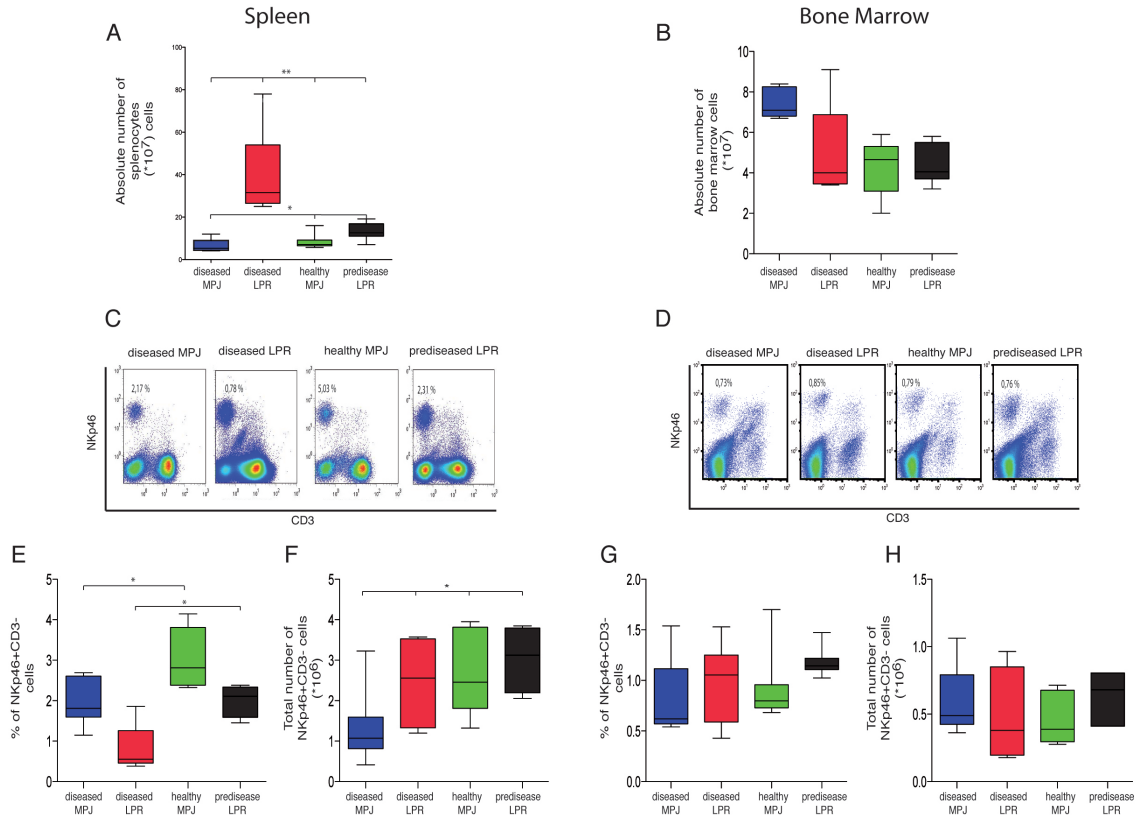

Figure R1 - Analysis **of absolute and relative NK cell numbers in bone marrow and spleen of healthy and diseased SLE-like mice**

A,B) Spleens and bone marrow (tibiae and femur) were isolated from mice, and the total number of cells from both spleen (a) and bone marrow (b) were counted using a haematocytometer. Freshly isolated mouse splenocytes and bone marrow cells were stained with anti-NKp46, -CD45 and -CD3 mAb. C,D) Representative flow cytometric density plots show the proportion of NK cells in the four mouse groups in both spleen (c) and bone marrow (d). E,F,G,H) Cumulative data representative of the mean percentage (e,g) and total number of $NKp46^{+}CD3^{-}$ cells (f,h) in both spleen and bone marrow of stained NK cells in all four groups. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 4 to 8 mice/group in three to six independent experiments; *$p<0.05$).

1.2 Natural killer cell surface marker expression

To determine whether the SLE-like environment affects NK cell maturation, we analyzed the expression of developmental markers in fresh NK cells derived from BM and spleen. NK cell development can be defined as a five- or six-step process, based on the expression pattern of cell surface markers [9, 29]. Expression of the IL-2 and IL-15 receptor γ chain (CD122) marks NK cell commitment in BM; CD122, as well as NKG2D and NK1.1 (not expressed in the MRL strain) are the earliest known NK cell markers [9]. Integrins such as CD49b (α2 integrin),

CD11b (Mac-1) and CD43 (leukosialin), which are upregulated on and correlate with phenotypically mature NK cells, are also common developmental markers for these cells. Gated NKp46^{+}CD3^{-} cells were analyzed for expression of these markers and of other known NK cell surface markers, such as CD2 (LFA-2) and CD11a (LFA-1) which partake in the formation of the NK cell immunological synapse [104].

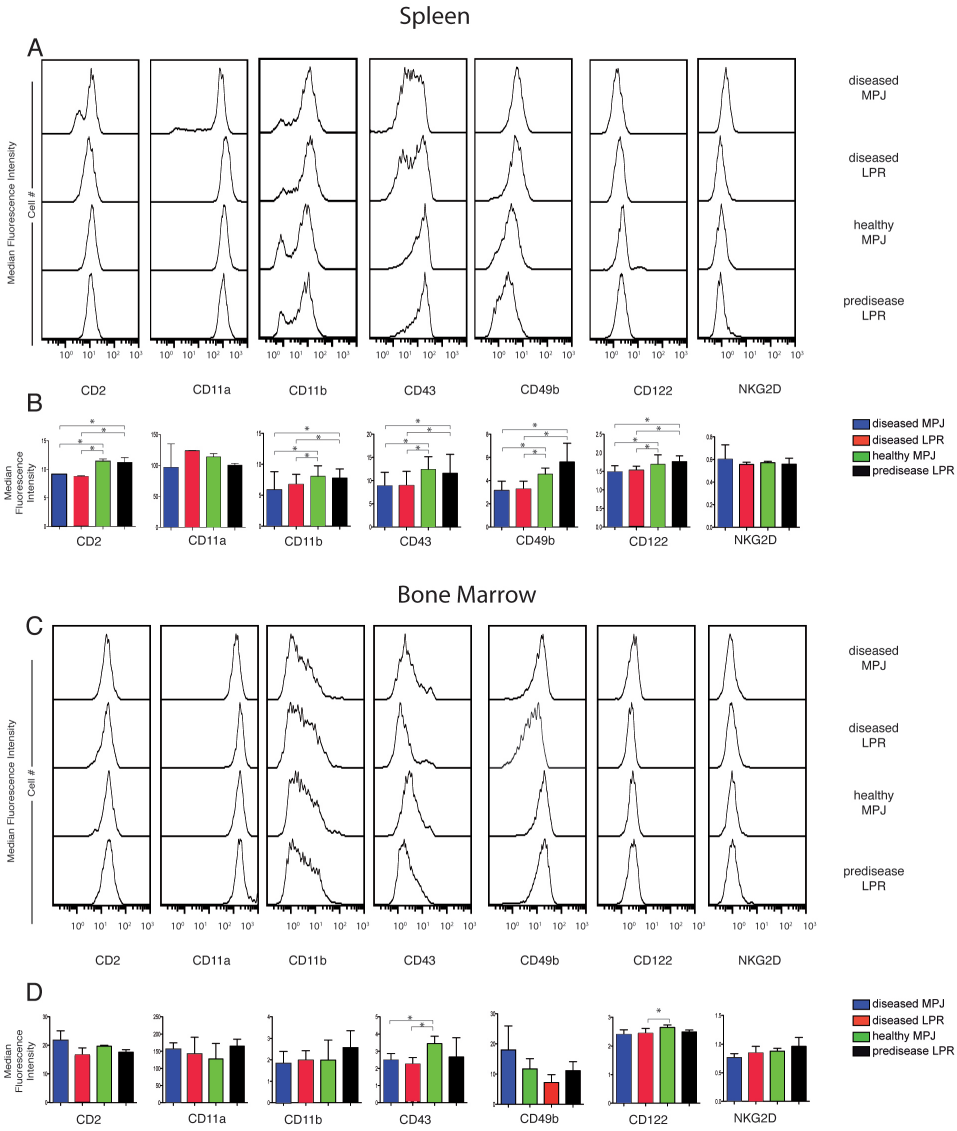

Figure R2 - Cell surface analysis of mature NK cell markers in bone marrow and spleen of healthy and diseased SLE-like mice

A,C) NK cells were gated as CD45^{+}NKp46^{+}CD3^{-} and stained for various NK cell markers. A representative staining for each marker is shown both in spleen (a) and bone marrow (c). Gates were set using unstained or nonspecific isotype mAb controls (not shown). B,D) The median fluorescence intensity for each markers was analyzed for both spleen (b) and bone marrow (d) samples. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 4 to 8 mice/group in three to six independent experiments; *$p<0.05$).

CD2, CD11b, CD43, CD49b, and CD122 were significantly downregulated in spleen NK cells from diseased LPR and MPJ mice compared to their healthy counterparts **(Figure R.2)**. In BM, CD122 and CD43 were downregulated only in diseased MPJ and LPR mice compared to healthy MPJ but not to prediseased LPR mice. NKG2D and CD11a levels were unchanged in BM and spleen NK cells from all mouse groups **(Figure R.2)**.

1.3 Immature NK cells accumulate in the spleen of diseased SLE-like mice

Based on the decrease in mature NK cell markers on peripheral mature NK cells, we postulated a defect in NK cell differentiation in the periphery. NK precursor cells (NKP) are thought to arise in BM; they can differentiate fully and mature in BM, or travel to other organs and complete their differentiation in situ. Indeed, NKP cells have been reported in peripheral organs of mice, including spleen [31]. NK cell differentiation is a complicated process that begins in the BM with the generation of NKP cells, which are $CD122^{+}Lin^{-}CD49b^{-}NKp46^{-}$. These cells then follow a developmental pattern that gives rise to immature NK (iNK) cells in which they begin to express some but not all NK-specific developmental markers: $CD122^{+}Lin^{-}CD49b^{-}NKp46^{-}$ (Stage 1 - NKP) → $CD122^{+}Lin^{-}NKp46^{+}CD49b^{-}$ (Stage 2) → $CD122^{+}Lin^{-}NKp46^{+}CD49b^{+}$ (Stage 3) [1, 31, 105, 106]. After Stage 3, NK cells begin to express NK cell marker CD11b and become $CD122^{+}Lin^{-}NKp46^{+}CD49b^{+}CD11b^{+}$ (Stage 4), and are considered Stage 4, mature NK cells. Analysis of total NKP and iNK cell numbers in BM and spleen of healthy and diseased LPR and MPJ mice, showed accumulation of Stage 1, Stage 2 and Stage 3 cells in spleen but not in BM of diseased LPR and MPJ mice compared to healthy MPJ and prediseased LPR mice **(Figure R.3)**.

1.4 $CD11b^{High}CD27^{Low}$ NK populations are greatly reduced in diseased SLE-like mice

NK cell maturation can be further subdivided into another four-stage process, dependent on CD27 and CD11b expression [105]. Studies have shown a process in which the NK cell maturation pattern correlates with progressive acquisition of NK cell effector functions in mature NK cells: $CD11b^{Low}CD27^{Low}$ → $CD11b^{Low}CD27^{High}$ → $CD11b^{High}CD27^{High}$ → $CD11b^{High}CD27^{Low}$.

Freshly isolated BM and spleen cells were gated in $NKp46^{+}CD3^{-}$ and analyzed for CD11b and CD27 expression. We observed a severe reduction in $CD11b^{High}CD27^{Low}$ NK cells in spleens from diseased LPR and MPJ compared to those from healthy LPR and MPJ mice; this correlated with an increase in $CD11b^{Low}CD27^{High}$ NK cells in diseased LPR and

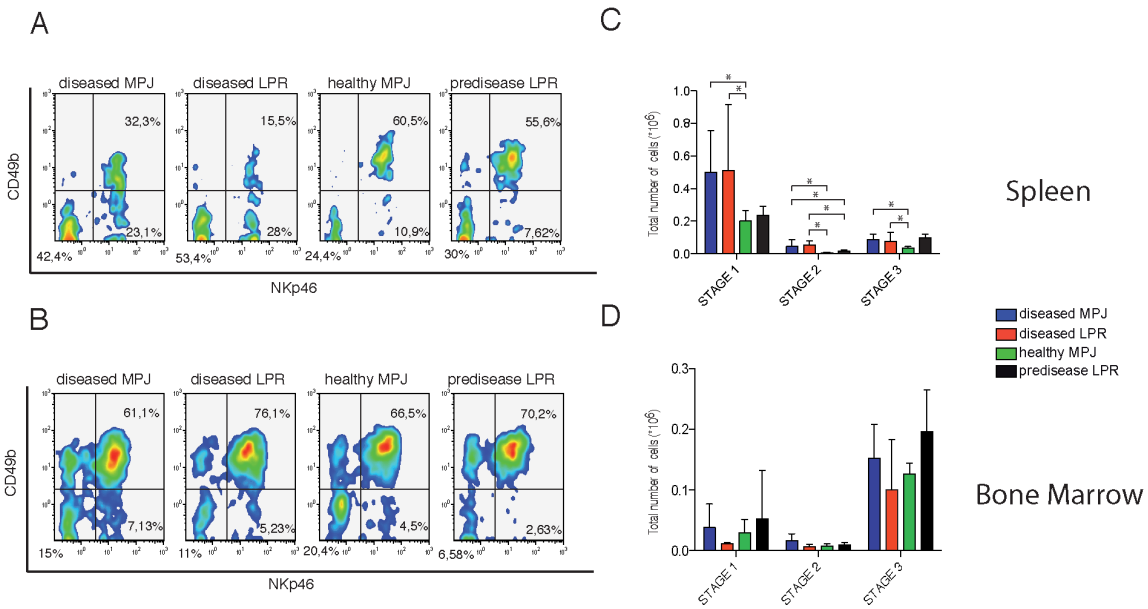

Figure R3 - Analysis of NKP and iNK cells in bone marrow and spleen of healthy and diseased SLE-like mice

A,B) Representative flow cytometric density plots show the proportion of $CD122^{+}LIN^{-}$ cells. Plots show proportions of Stage 1 ($CD122^{+}LIN^{-}NKp46^{-}CD49b^{-}$), Stage 2 ($CD122^{+}LIN^{-}NKp46^{+}CD49b^{-}$) and Stage 3 ($CD122^{+}LIN^{-}NKp46^{+}CD49b^{+}$) iNK cell groups in spleen (a) and bone marrow (b). C,D) Absolute numbers were calculated of total Stage 1, 2, and 3 iNK cells. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 4 to 8 mice/group in three to six independent experiments; *$p<0.05$).

MPJ mouse spleens **(Figure R.4A)**. There were no significant differences in CD11bCD27 subsets in NK cells from fresh BM of any mouse tested **(Figure R.4B)**.

1.5 Spleen and BM NK cells show slight conjugation defects

To assess whether BM and spleen NK cells in a SLE environment also have functional defects, we performed conjugation and CD107a degranulation assays using 6-day-cultured IL-15-activated NK cells from healthy MPJ and diseased LPR mice. NK cells were not activated with IL-2, as LPR mice do not respond well to IL-2 [107]. Conjugation at short intervals (0, 5, 10 and 20 min) was tested using YAC-1 NKG2D ligand-expressing target cells. There were no differences in the conjugation capacity of BM IL-15-activated NK cells between diseased LPR and healthy MPJ mice. Spleen IL-15 activated NK cells from diseased LPR mice showed conjugation defects at 20 min **(Figure R.5A)**.

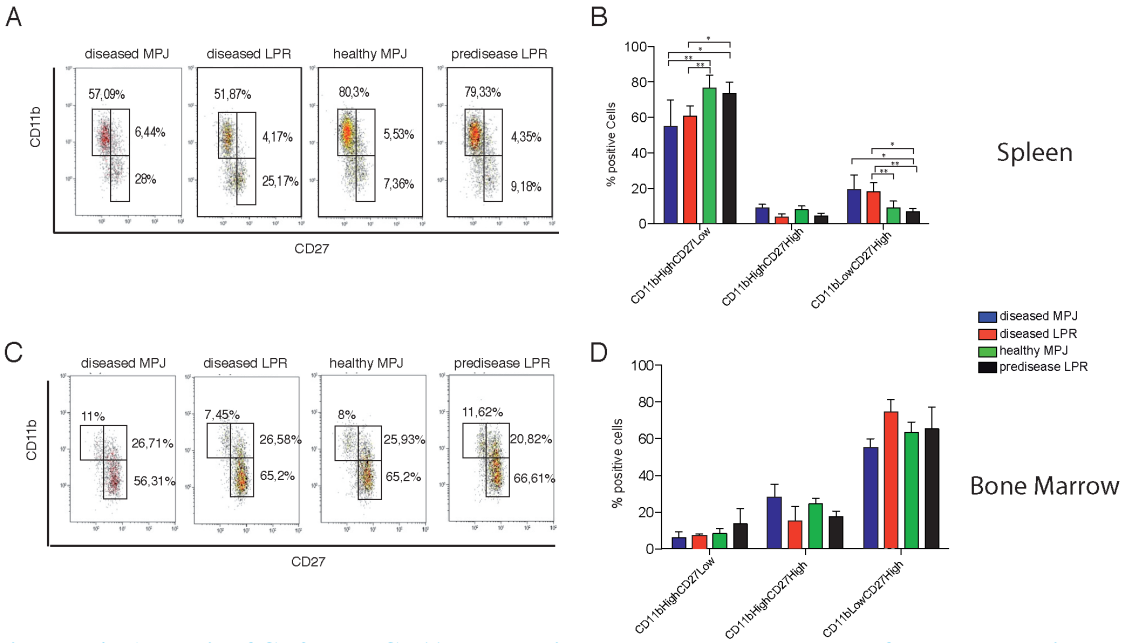

Figure R4 - Analysis of CD27 and CD11b subsets in bone marrow and spleen of healthy and diseased SLE-like mice

Freshly isolated mouse splenocytes and BM cells were stained with anti-NKp46 and -CD3 mAb and analyzed for CD11b and CD27 expression. Representative flow cytometric density plots show the percentage of CD11bCD27 NK cell subpopulations in splenocytes (A) and BM cells (C). Percentages were calculated of $CD11b^{Low}CD27^{High}$, $CD11b^{High}CD27^{High}$, and $CD11b^{High}CD27^{Low}$ cells for splenocytes (B) and BM cells (D). Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 4 to 8 mice/group in three to six independent experiments; $^{**}p<0.01$, $^{*}p<0.05$).

1.6 BM and spleen NK cells from diseased LPR mice degranulate less CD107a than healthy MPJ mice

Degranulation of the lysosome-associated membrane protein-1 CD107a (LAMP-1) is upregulated on the NK cell surface following stimulation with target cells able to induce a cytolytic response. As CD107a expression correlates with cytokine secretion and NK cell-mediated lysis of target cells, we used this as a marker to determine whether NK cells from SLE mice suffer from cytolytic defects [108]. As target cells, we used CHO (which express a homologue of H2-Dd), RMA/S (which express low levels of MHC class I), and YAC-1 cells (which express NKG2D ligands). After stimulation with all three target cell lines, both BM and spleen IL-15-activated NK cells from diseased LPR mice showed significant decreases in $CD107a^{+}$ cells compared to those from healthy MPJ mice **(Figure R.5B)**.

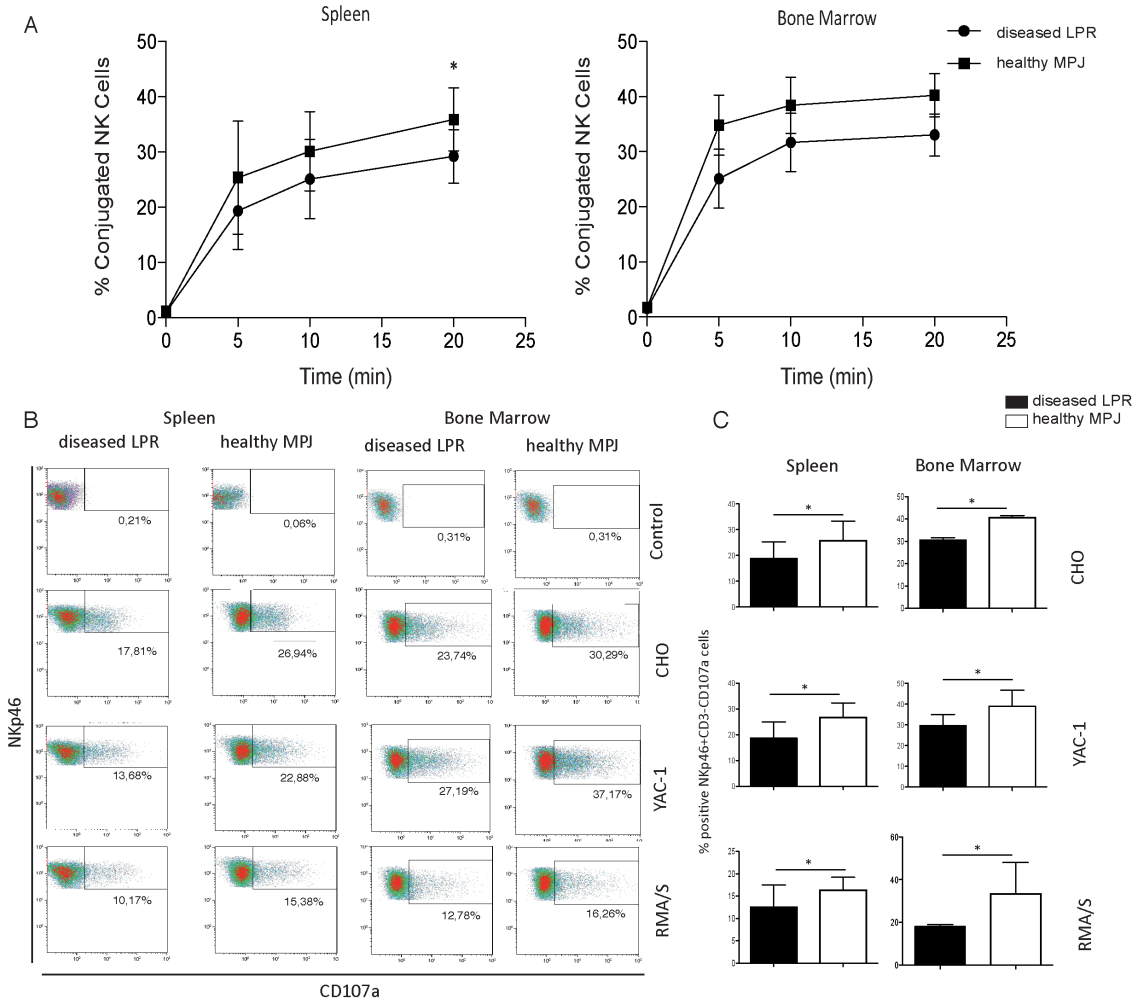

Figure R5 - CD107a and conjugation assays on IL-15 activated splenocytes and bone marrow cells from healthy and diseased SLE-like mice

A) Six-day IL-15-activated spleen or BM NK cells were tested in a four-point conjugation assay against YAC-1 target cells at a 2:1 target:effector ratio. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 3 mice/group in three independent experiments; *p<0.05). B,C) Spleen or BM NK cells, activated as above, were tested against the CHO, RMA/S and YAC-1 target cell lines at a 2:1 target:effector ratio in a 4 h CD107a degranulation assay. Representative stainings are shown (B); data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 3 to 5 mice/group in three independent experiments; *p<0.05)

1.7 Characterization of kidney infiltrating NK and CD3 cells

Having established the existence of NK cell maturation defects in BM and spleen from diseased SLE-like mice, we decided to analyze via flow cytometry, the lymphocyte populations of kidneys in our four mice models. To date, the only information available on kidney infiltrating lymphocytes has been via immunohistochemical stainings, and to our knowledge, no

complete analysis of these populations had been done comparing healthy and diseased kidneys during an active disease state.

Freshly ficoll-isolated lymphocytes from kidneys were stained for CD45, CD3, CD4, CD8, NKp46, and various NK cell surface markers, and gated via the exclusion of dead cells and doublets **(Figure R.6A,B)**. Our analysis showed an increase in the absolute number of

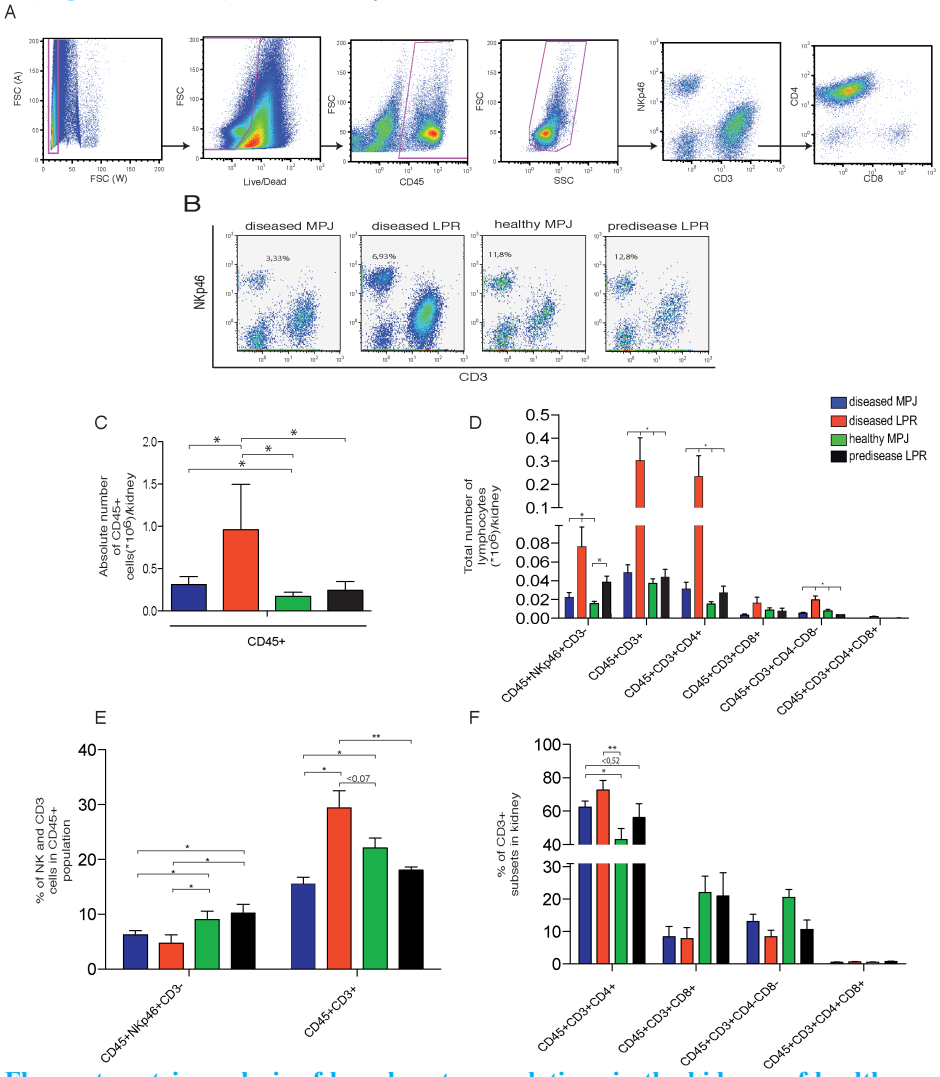

Figure R6 - Flow cytometric analysis of lymphocyte populations in the kidneys of healthy and diseased SLE-like mice

Gating strategy for Ficoll-isolated kidney lymphocytes. A) Representative gating strategy for acquisition of Ficoll-isolated kidney lymphocytes. Briefly, cells were gated via doublet discrimination, followed by live/dead discrimination, followed by CD45 positivity, followed by debris discrimination. Cells were then gated on $CD3^{+}NKp46^{-}$ subsets for analysis of CD4 and CD8 subsets. B) Representative flow cytometric density plots show the proportion of NK cells in the four mouse groups C,D) Cumulative data representative of the total number of $CD45^{+}$ cells (c) and NK and CD3 subpopulations (d). E,F) Cumulative data representative of the mean percentage of NK and CD3 cells (e) and of CD3 subpopulations (f). Gates were set using unstained or nonspecific isotype mAb controls (not shown). Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 4 to 8/group; three to four independent experiments, **p<0,01, *p<0.05).

$CD45^+$ cells infiltrating the kidneys of diseased LPR and MPJ mice compared to healthy and prediseased mice **(Figure R.6C)**. We also show a significant increase in the total numbers of NK cells only in the kidneys of diseased LPR mice compared to all other groups **(Figure R.6D)**. Prediseased LPR mice showed higher levels of NK cells compared to healthy MPJ mice. The total number of $CD45^+CD3^+CD4^+$, $CD45^+CD3^+CD8^+$, $CD45^+CD3^+CD4^-CD8^-$ cells was also found to be increased only in diseased LPR, but not diseased MPJ kidneys, compared to healthy and predisease controls **(Figure R.6D)**.

1.8 Phenotypic characterization of infiltrating kidney NK cells

An analysis of the phenotypic characterization of kidney infiltrating NK cells showed, in contrast to spleen and BM, no differences in NKG2D, CD122, CD2 or CD49b expression in diseased mice compared to their healthy counterparts **(Figure R.7B)**. CD11b and CD43 levels were nonetheless increased in diseased MPJ and LPR NK cells compared to prediseased LPR and healthy MPJ NK cells **(Figure R.7B)**.

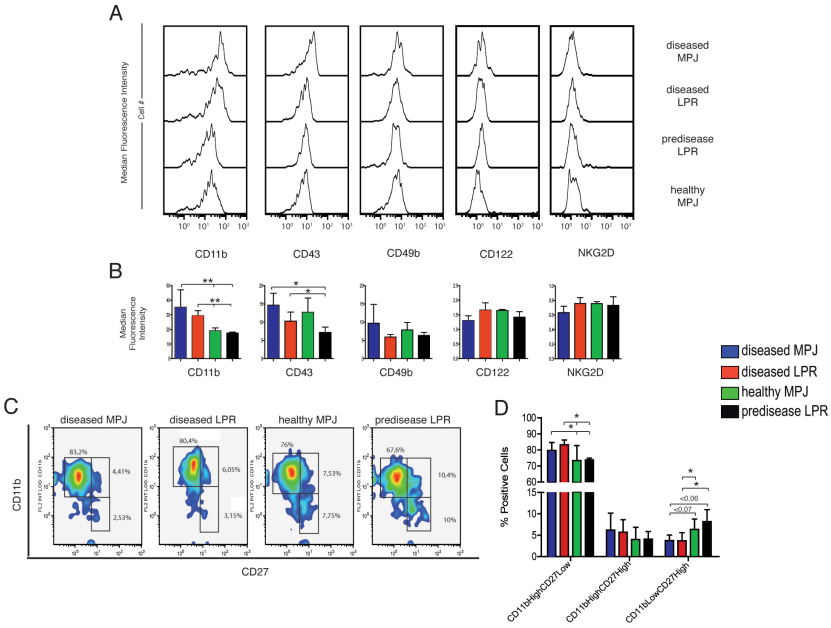

Figure R7 - Phenotypic analysis of kidney infiltrating NK cells in healthy and diseased SLE-like mice
A) NK cells were gated as $NKp46^+CD3^-$ and stained for various NK cell markers. A representative staining is shown for each marker. Gates were set using unstained or nonspecific isotype mAb controls (not shown). B) The median fluorescence intensity for each marker was analyzed. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 4 to 8 mice/group in three to six independent experiments; $^{**}p<0.01$, $^{*}p<0.05$). C) Representative flow cytometric density plots show the percentage of CD11bCD27 NK cell subpopulations D) Percentages of $CD11b^{Low}CD27^{High}$, $CD11b^{High}CD27^{High}$, and $CD11b^{High}CD27^{Low}$ cells were calculated. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 4 to 8 mice/group in three to six independent experiments; $^{*}p<0.05$).

The CD11b expression increase in diseased LPR and MPJ mice also correlated with significantly larger percentages of $CD11b^{High}CD27^{Low}$ cells compared to $CD11b^{High}CD27^{High}$ and $CD11b^{Low}CD27^{High}$ cells **(Figure R.7C,D)**. We observed a clear tendency in $CD11b^{Low}CD27^{High}$ cell accumulation in healthy MPJ and prediseased LPR mice compared to diseased LPR and MPJ mice.

1.9 Functional characterization of infiltrating NK cells in kidney

To ascertain whether infiltrating NK cells showed the same defective functional characteristics as those in periphery, we tested for the ability of these cells to produce IFN-γ after PMA/ionomycin stimulation. Significant differences in the percentages of IFN-γ producing cells in kidneys were found in the NK, but not CD4 or CD8 compartments. Kidney NK cells from diseased MPJ and LPR mice had a larger percentage of cells that responded to PMA/ionomycin; furthermore, these cells secreted more IFN-γ (as measured by MFI) than healthy MPJ or prediseased LPR **(Figure R.8A,C)**. Moreover, the difference in the percentage of IFN-γ producers was found in $CD11b^{High}CD27^{Low}$ cells, but not in the $CD27^{High}$ subset **(Figure R.8B)**.

1.10 Organ-specific induction of pSTAT5 in NK cells

As NK cells suffer from impaired differentiation and maturation in spleen from diseased mice, but not in BM or kidney (where NK cells appear to have a more active phenotype), we postulated that the STAT5 phosphorylation pathway might be altered in diseased NK cells. STAT5 is a member of the STAT family of proteins, which signal via the JAK/STAT pathway. STAT5 is central to modulation of the biological response to cytokines such as IL 15, and STAT phosphorylation levels were recently correlated to SLE activity in humans [109, 110]. To determine whether NK cells from these organs have a differential capacity to phosphorylate STAT5, we used murine IL-15 to stimulate fresh kidney lymphocytes and single cell suspensions from spleen and BM from of both healthy and diseased LPR and MPJ mice. FACS analysis of BM and spleen showed no differences between the groups in the STAT5 expression levels after IL-15 stimulation **(Figure R.9)**. In contrast, kidney NK cells from diseased LPR and MPJ mice showed higher pSTAT5 phosphorylation levels than healthy MPJ and prediseased LPR mice after IL-15 stimulation **(Figure R.9)**. Furthermore, kidney NK cells were seen to be able to phosphorylate more STAT5 than their spleen and BM counterparts.

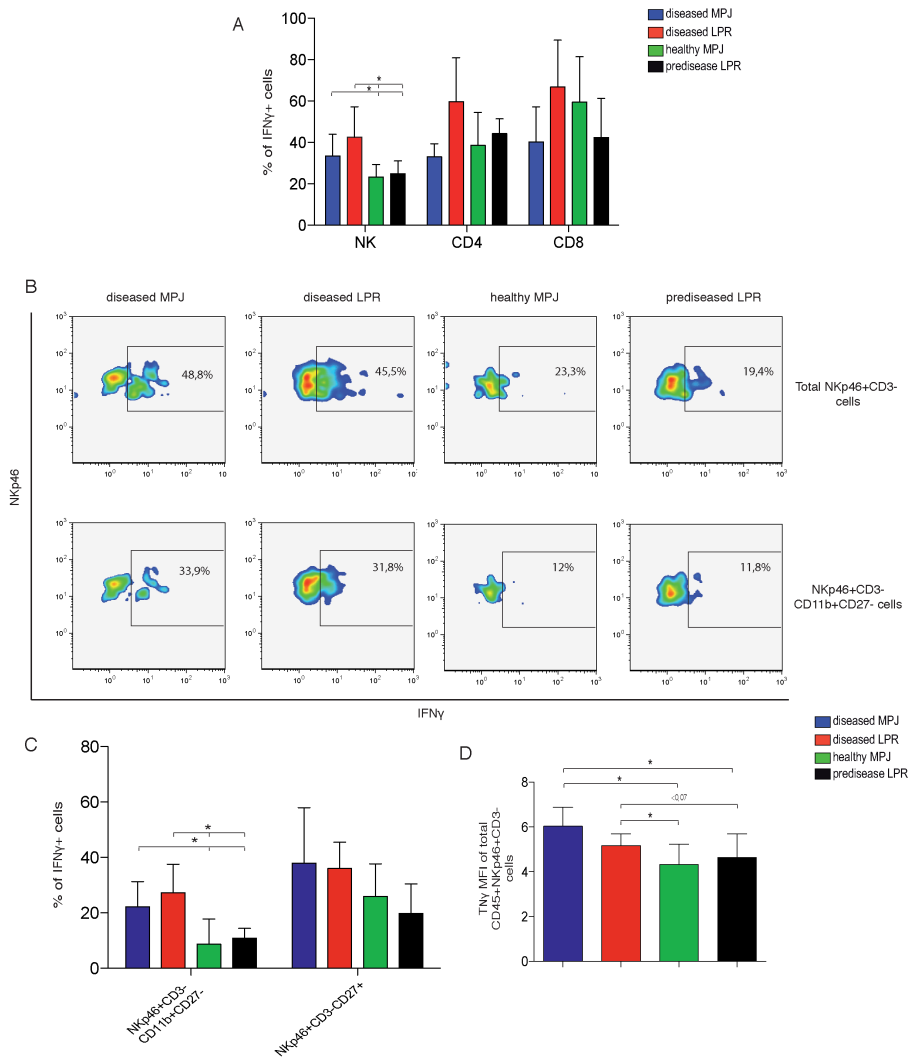

Figure R8 - Production of IFNγ after PMA/ionomycin stimulation of kidney infiltrating NK cells in healthy and diseased SLE-like mice

Ficoll-isolated kidney lymphocytes were incubated with PMA, ionomycin and BFA, and stained with anti-NKp46, -CD45, -CD3, -CD4, -CD8, -CD11b, -CD27, and -IFNγ mAb (4 h). A) Percentages of $CD45^+NKp46^+CD3^-IFN\gamma^+$, $CD45^+NKp46^-CD3^+CD4^+IFN\gamma^+$, and CD45+$NKp46^-CD3^+CD8^+IFN\gamma^+$ cells. B) Representative flow cytometric density plots show the percentage of gated $CD45^+NKp46^+CD3^-$ and $CD45^+NKp46^+CD3^-CD11b^+CD27^-$ cells positive for IFNγ. C) Percentages of $CD45^+NKp46^+CD3^-CD11b^+CD27^-IFN\gamma^+$ and $CD45^+NKp46^+CD3^-CD27^+IFN\gamma^+$ cells. D) Total MFI of $CD45^+NKp46^+CD3^-IFN\gamma^+$ cells. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 4 to 6 mice/group in three to four independent experiments; *$p<0.05$).

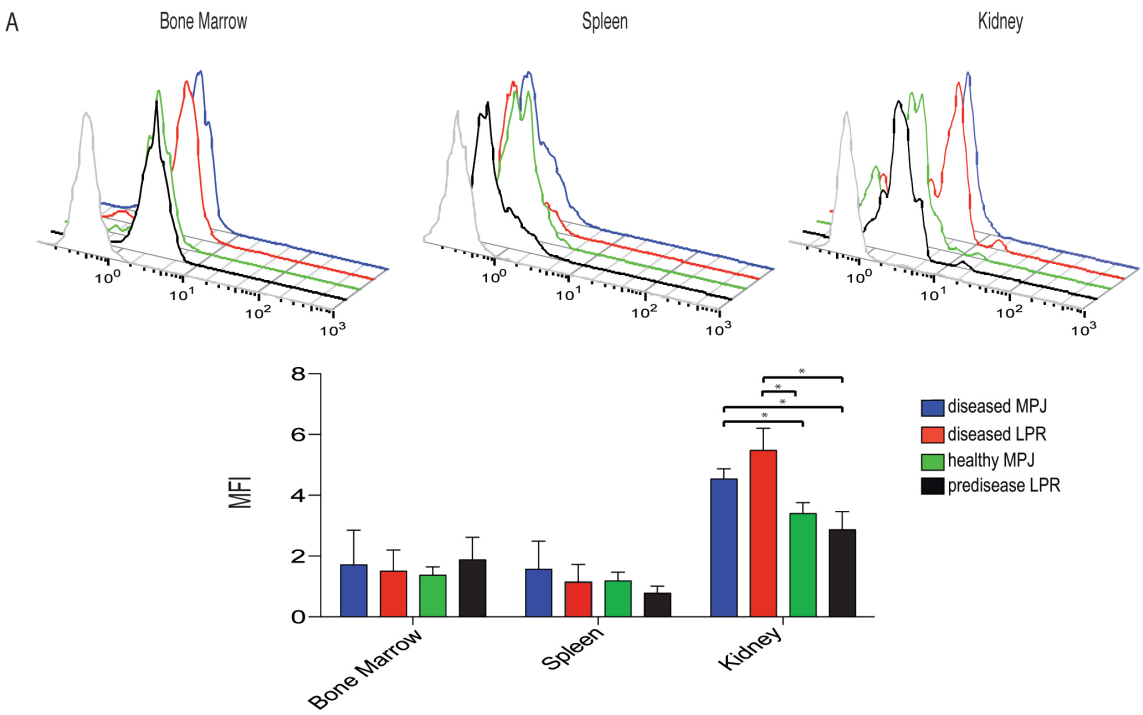

Figure R9 - STAT5 phosphorylation of NK cells from healthy and diseased SLE-like mice after IL-15 stimulation

Fresh single cell suspensions of spleen, BM, and Ficoll-isolated kidney lymphocytes were activated with recombinant murine IL-15 (15 min), fixed, permeabilized and stained with anti-NKp46, -CD45, -CD3, and -phospho-STAT5 mAb. A) Representative flow cytometric histograms show pSTAT5 expression on gated $CD45^+$ NK cells for diseased MPJ (blue line), diseased LPR (red line), healthy MPJ (green line) and prediseased LPR mice (red line). Isotype control staining (grey line) is shown in each panel. B) NK cells were gated for $CD45^+NKp46^+CD3^-$ and the median fluorescent intensities were calculated for total pSTAT5 expression. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 4/group in four independent experiments; *$p<0.05$).

1.11 IL-15, but not IL-12, is increased in the kidneys of diseased SLE-like mice

To elucidate possible mechanisms which might be implicated in the increase of mature NK cells in the kidneys of diseased mice, we decided to analyze the levels of IL-15 and IL-12, two cytokines, which as previously stated, are known for their importance in NK cell development and activation. The levels of IL-15 were measured using a IL-15/IL-15 receptor conjugated ELISA kit, as it has been reported that IL-15 in vivo is mainly secreted both in mice and humans in complex with its unique receptor, IL-15Rα, produced by either dendritic cells, monocytes, fibroblasts, and/or macrophages [111, 112]. We show a generalized increase in the levels of IL-15 in the kidneys of diseased SLE-like mice compared to their controls, but not of IL-12 **(Figure R.10)**. Furthermore, we also analyzed the levels of CXC3CL1, a chemokine known to play a role in NK cell migration to inflamed organs such as lung and skin [37]. Via ELISA we were capable

of noticing only an increase in the levels of CXC3CL1 only in the kidneys of prediseased LPR mice, but not in the other groups, which showed similar expression levels of CXC3CL1 **(Figure R.10)**.

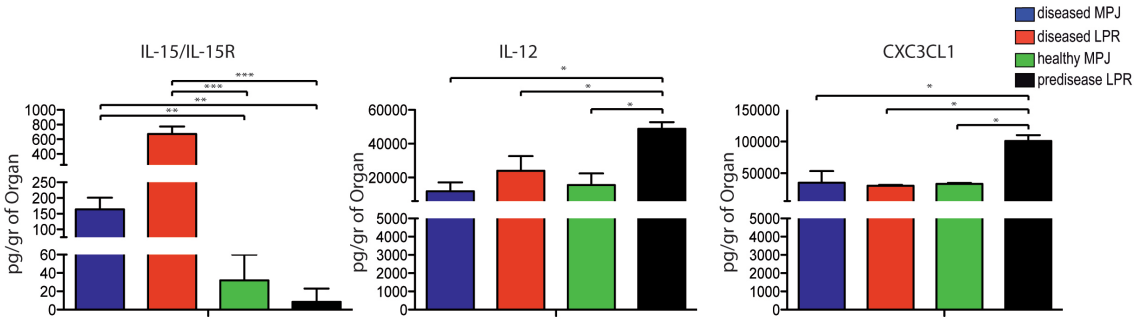

Figure R10 – IL-15 but not IL-12 is increased in diseased SLE-like mice kidneys

Total levels of IL-15, CXC3CL1, and IL-12 were measured from the supernatants collected from kidney tissue homogenates. The final concentration of cytokine was then normalized to the total weight of each organ. A representative experiment of each ELISA is shown (mean ± SD; n = 2/group in two independent experiments). Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 4/group in four independent experiments; ***p<0,001, **p<0,01, *p<0.05).

Part 2 NKG2D Ligand Expression the MRL mouse strain

2.1 Rae-1 expression in MPJ, LPR and C57BL/6 mouse kidneys

NKG2D ligand involvement in SLE pathogenesis, particularly in the glomerulonephritic process, has not been studied. To assess whether the MRL lupus-prone genetic background causes NKG2D ligand upregulation and thus participates in glomerulonephritis onset, we tested NKG2D ligand expression in MRL mouse kidney. C57BL/6 (BL6) mice were used as a genotype-negative control strain, in which no ligand expression was anticipated. We tested antibodies to various NKG2D ligands, including H60 (not expressed in BL6 mice), Rae1, and MULT-1, to assess their validity for immunohistochemistry [113, 114]. Only the anti-Rae-1γ antibody AF1136 stained appropriately [95]. In our experiments, Rae 1γ stained clearly in the glomeruli of 3-month-old female healthy MPJ and diseased LPR mice, but not in equivalent BL6 controls; Rae-1γ was also stained in glomeruli of 9-week-old prediseased LPR and 1 year-old diseased MPJ mice **(Figure R.11A)**. Analysis of the intensity of Rae-1γ staining showed a significantly more intense staining in the glomeruli of diseased LPR and MPJ mice than in their healthy counterparts **(Figure R.11B)**.

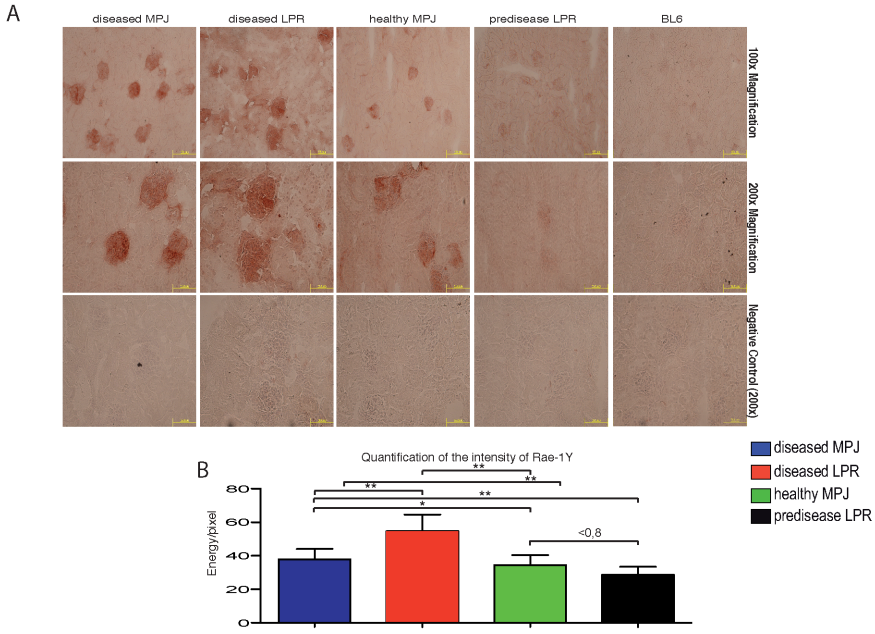

Figure R11 - Immunohistochemical analysis of Rae-1γ expression in healthy and diseased SLE-like mice

Analysis of Rae-1γ. A) Immunohistochemical analysis of Rae-1γ expression in kidney tissue cryosections from LPR, MPJ, and C57BL/6 mice at various ages. Specific staining (red) is observed in the glomeruli of all mice of the MRL genotype, whereas staining is absent in C57BL/6 controls. Representative images of kidneys from five mice per group. B) Quantification of Rae-1γ staining in MRL mouse glomeruli. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 5/group; **p<0.0001 *p<0.0025).

2.2 Rae-1γ and MULT-1 expression are limited and specific to glomeruli of the MRL genotype

To confirm specific Rae-1γ expression in the glomeruli and to show that all glomeruli expressed this NKG2D ligand, we used immunofluorescence analysis with a combination of antibodies to Rae-1γ and to synaptopodin, a marker for differentiated glomerular cells [115, 116]. Rae-1γ expression was specific to synaptopodin-expressing cells and all glomeruli were Rae-1γ-positive **(Figure R.12)**.

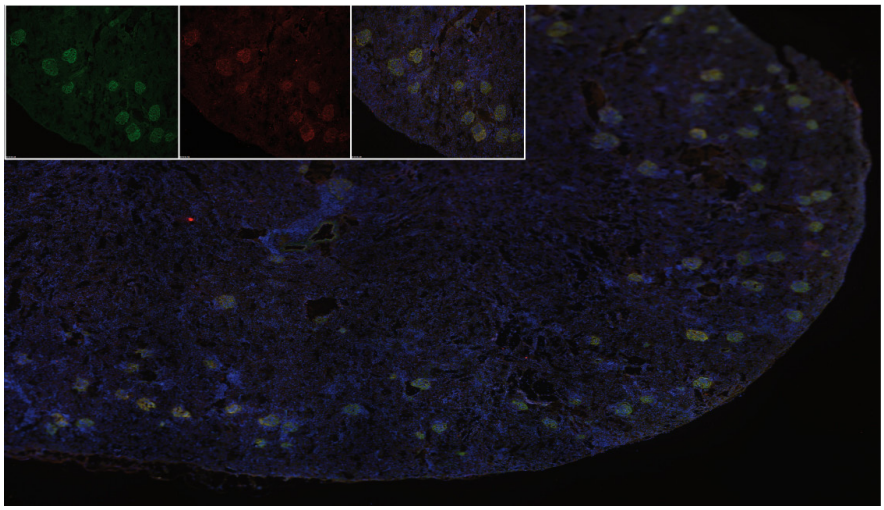

Figure R12 - Representative immunofluorescent staining of Rae-1γ in a whole section from diseased LPR kidney

Cryosections of diseased LPR kidney (10 μm) were co-stained with rabbit anti-mouse synaptopodin (green) and goat anti-mouse Rae-1γ antibody (red), followed by appropriate Alexa-conjugated secondary antibodies and DAPI (blue). All synaptopodin-positive cells corresponded to Rae-1γ-positive cells, indicating glomerulus-specific expression of this NKG2D ligand. Negative controls using secondary antibody alone are not shown.

We evaluated expression of the other NKG2D ligand families on the MRL background. Using an anti-MULT-1 antibody of known efficiency in immunofluorescence analysis, we tested kidney sections from MRL and BL6 mice [99]. Confocal analysis of cryosections showed strong glomerulus-specific MULT-1 expression in MRL but not BL6 mice **(Figure R.13)**. No anti-H60 antibodies tested showed positive staining.

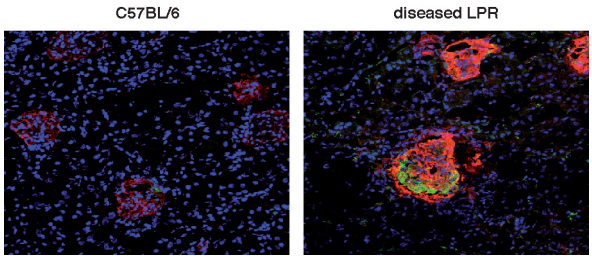

Figure R13 - Confocal analysis of synaptopodin and MULT-1 expression in C57BL/6 and diseased LPR kidney sections

Kidney cryosections were stained with rabbit anti-mouse synaptopodin (red) and anti-rat MULT-1 (green) followed by appropriate secondary antibodies and DAPI (blue). MULT-1 expression was absent in the C57BL/6 strain.

To evaluate whether Rae-1γ is commonly expressed in glomeruli of other mice prone to SLE-like disease and not in healthy strains, we used immunohistochemical analysis of Rae-1γ in kidney sections of diseased NZBxNZW(F1) and healthy 3-month-old BALB/c mice. NZBxNZW(F1) is a SLE-like murine model whose lupus susceptibility is contributed by both the NZB and NZW genomes. These mice were positive for Rae-1γ expression in glomeruli, while no Rae-1γ was observed in the glomeruli of BALB/c mice **(Figure R.14)**.

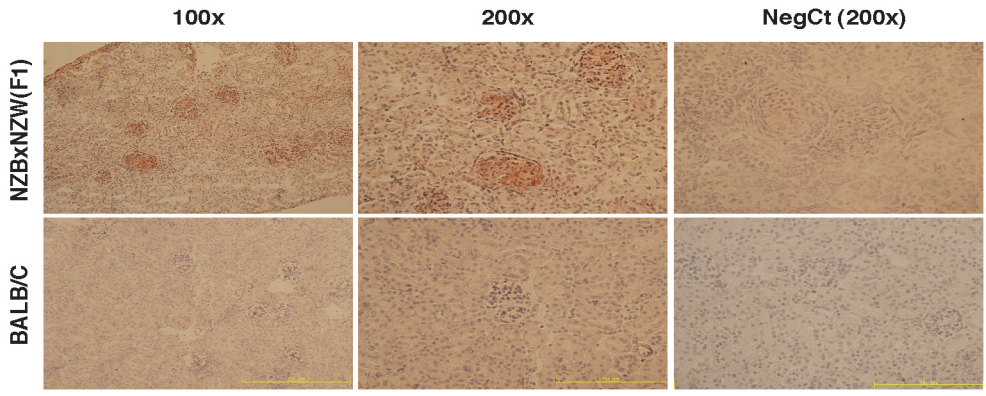

Figure R14 - Representative immunohistochemical staining of Rae-1γ in glomeruli of NZBxNZW(F1) and BALB/c mice.

Rae-1γ (red) staining shown in the glomeruli of diseased NZBxNZW(F1) mice, while hematoxylin counterstain (blue) shows cell nuclei. No Rae-1γ staining was observed in BALB/c mice.

2.3 NKp46 reactivity in MRL mouse tissues

Study of NK cell infiltration in the MRL mouse model required a functional antibody for immunohistochemical studies, as MRL mice are NK1.1-deficient and no NK cell-positive immunohistochemical marker has been reported to date for this model. Previous studies showed NK cell staining in BL6 mice using the 4D11 (anti-Ly-49G2) mAb [117, 118]. We nonetheless detected no positive staining with this mAb in MRL spleen sections (not shown). We thus tested antibodies to other NK cell markers, and found that the anti-CD335 (NKp46) antibody (AF2225) stained spleen sections of BL6 and MRL mice **(Figure R.15)**. This is first antibody reported for immunohistochemical identification of NK cells in the MRL model.

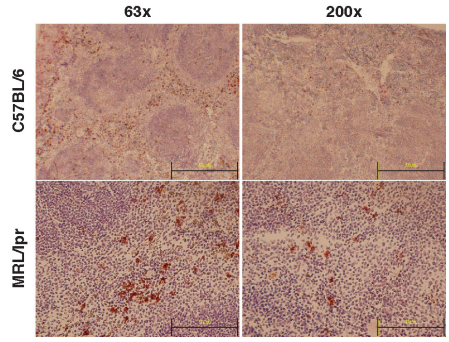

Figure R15 - Representative immunohistochemical staining of NKp46 in C57BL/6 and MRL spleen sections.

Red membrane staining shows NK cells; blue counterstain (hematoxylin) shows cell nuclei. Negative controls not shown.

2.4 NKp46, CD4 and CD8 infiltration in MRL and BL6 mouse glomeruli

To ascertain whether NKp46$^+$, CD4$^+$ and CD8$^+$ cell infiltration correlates with glomerular infiltrates, we used immunohistochemical techniques to analyze the percentage of glomeruli that showed NK, CD4 or CD8 cell infiltration. Diseased LPR mice showed higher levels of NKp46$^+$, CD4$^+$ and CD8$^+$ cell infiltration in glomeruli than all other mouse groups. Glomeruli of diseased MPJ and prediseased LPR mice also showed significantly more NKp46$^+$, CD4$^+$ and CD8$^+$ cell infiltrates than healthy MPJ mice **(Figure R.16)**.

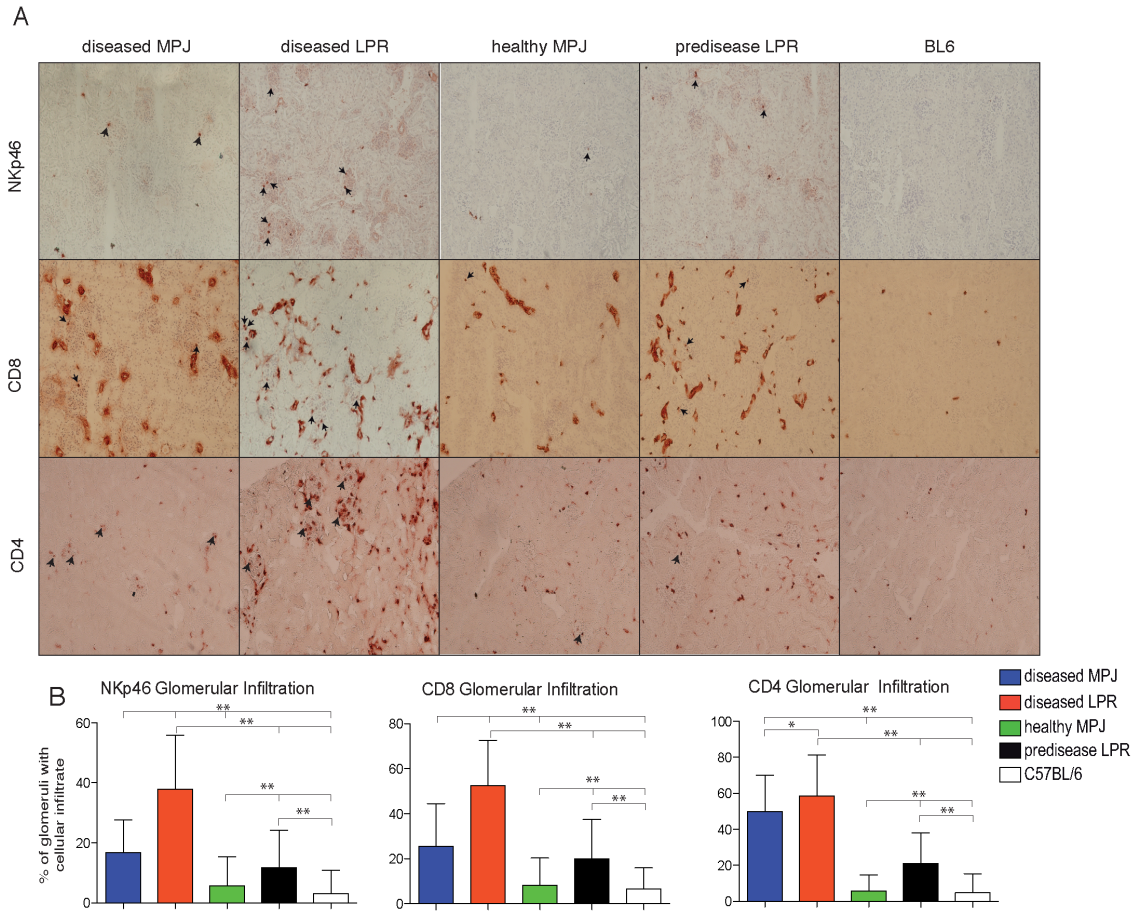

Figure R16 - Quantification of NKp46, CD4, and CD8 infiltrates in glomeruli.

Representative images of immunohistochemical staining of NK (NKp46), CD4 (CD4) and CD8 (CD8) cells in kidney tissue cryosections from MPJ, LPR, and C57BL/6 mice at various ages. Positive staining inside glomeruli (black arrows). B) Quantification of the percentage of glomeruli with cell infiltrates per field (x100). Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 5/group; 25 kidney sections/mouse, *p<0.001, **p<0.0001).

2.5 NKG2D ligand expression in kidneys of SLE patients with active disease

To ascertain if NKG2D ligands were also present in the kidneys of diseased SLE patients, we checked via immunohistochemistry, for the presence of the NKG2D ligands MICA and ULBP1 in six patients with active SLE with diagnosed Stage III or Stage IV lupus nephritis. Our analysis revealed only a slight staining of ULBP1 in one patient with Stage IV lupus nephritis **(Figure R.17)**.

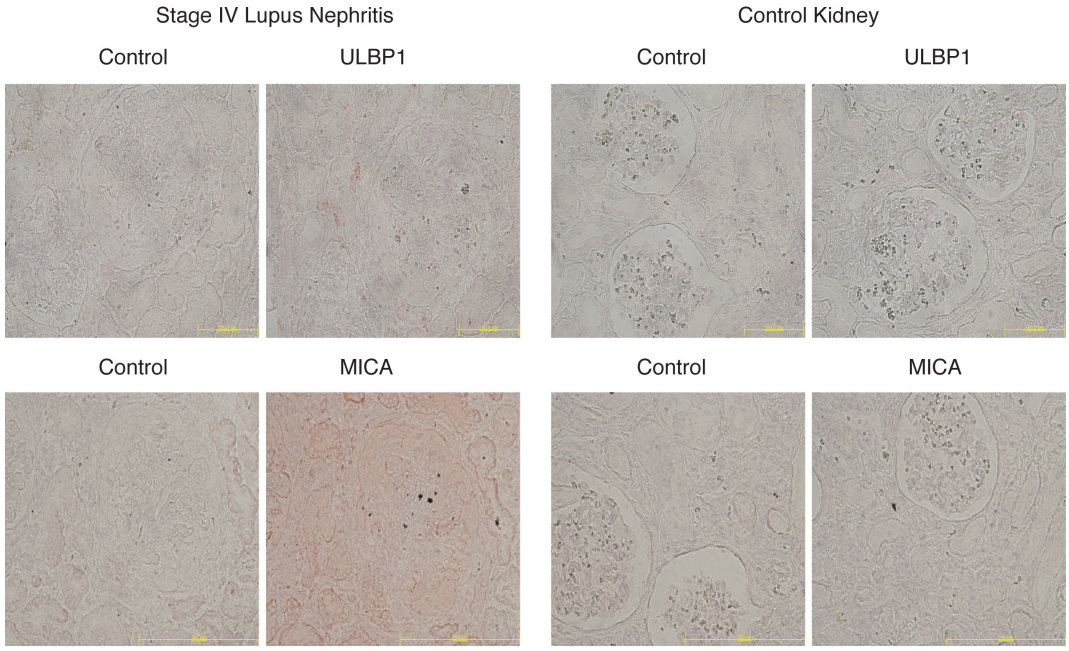

Figure R17 – Expression of NKG2D ligands in SLE patients with active disease.
Representative images of immunohistochemical staining of MICA or ULBP1 in kidney tissue formalin-fixed sections from patients with active SLE or healthy cohorts. Staining was only noticed in patients with active SLE and not healthy controls. Negative control antibody stains are shown for each staining in each group.

However four out of the six patients tested showed diffused positive staining for MICA in both in glomerular and peritubular endothelium **(Figure R.17 and Table R.1)**. No staining for either MICA or ULBP1 was had in control kidneys which were taken from healthy patient biopsies. These findings allow us to postulate that NKG2D ligands might indeed be upregulated in patients with active SLE, even though a more thorough investigation with more patients and a larger panel of NKG2D ligand antibodies must be performed.

Patient	Type of Nephropathy	ULBP1	MICA
1	Stage IV (Global-Chronic)	-	++
2	Stage III (Acute/Chronic)	-	-
3	Stage IV (Segmentated-Acute/ Chronic)	-	-
4	Stage IV (Global-Acute)	-	+
5	Stage IV (Global-Acute)	+	+++
6	Stage III (Acute)	-	++

Table R.1 – List of patients with active SLE and relative staining intensity for ULBP1 or MICA

Part 3 Study of NK cell maturation in sustained Rae-1 expressing mouse models

3.1 Systemic and local Rae-1ε expressing mouse models

To determine if high-levels of constitutive Rae-1 expression, as seen in the glomeruli of diseased SLE-like mice of the MRL genotype, could alter the maturation state of NK cells, we used two mice models which constitutively express Rae-1ε. One of these transgenic models expresses high levels of Rae-1ε systemically, via its control under the chicken β-actin gene and cytomegalovirus enhancer which is active in all tissues (FVB-187) **(Figure R.18A)**. The second model, expresses Rae-1ε under the control of a human involucrin gene promoter active only in

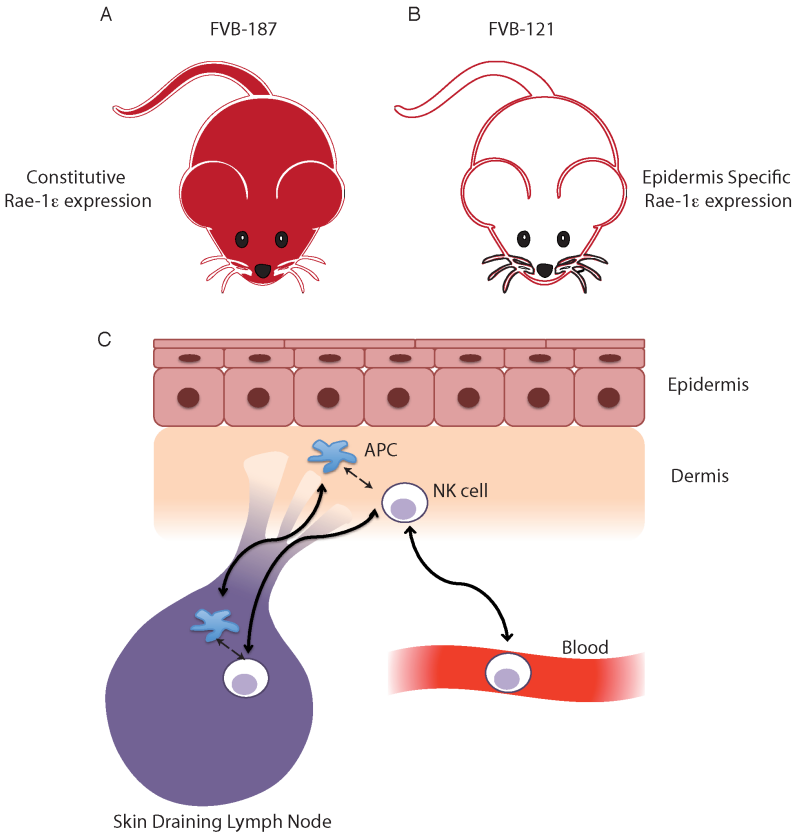

Figure R18 - Transgenic Rae-1ε protein expression from an epithelium-specific or globally active promotor

Diagram showing Rae-1ε expression (red) either in FVB-187 (a) or FVB-121 (b) mice. C) Factors in the epidermis can modulate NK cells present in skin draining lymph nodes. Epidermal factors, such as the increased levels of Rae-1ε in the epidermis in FVB-121 mice, can modulate NK cell responses either directly or indirectly. Direct interaction with the epidermis occurs via either circulating NK cells from the blood stream, or dermis resident NK cells. These cells can either interact with antigen presenting cells (APC), also located in the dermis. Indirect modulation can occur if circulating NK cells which are located in the skin draining lymph nodes make contact with APCs in situ.

squamous epithelium (FVB-121)[119] **(Figure R.18B)**. Using these two models, we checked if constitutive Rae-1 expression could modulate the levels of the CD11bCD27 NK cell subsets. Seeing as how the FVB-187 mice constitutively express Rae-1ε, we decided to specifically analyze peripheral NK cells present in the spleen. In FVB-121 mice, as Rae-1ε expression is limited only to the epidermis, we analyzed NK cells from skin draining lymph nodes, as NK cells present in these lymph nodes would be capable of interacting with the Rae-1ε present in the epidermis either directly, via circulating NK cells, or indirectly, via the interaction these NK cells could have with other lymphocyte groups, such as antigen presenting cells (APC) **(Figure R.18C)** [120-122] .

3.2 Systemic Rae-1 expression correlates with higher levels of CD11b^{High} cells

An initial analysis into the general expression levels of CD11b, showed a substantial increase in the MFI of CD11b in the NK cell compartment in both the spleen and skin draining lymph nodes of FVB-187 mice compared to their WT littermates **(Figure R.19D)**. No differences in the MFI of CD11b in NK cells was noted in FVB-121 mice compared to their WT littermates in the skin draining lymph nodes **(Figure R.19C)**. However, the analysis of the CD11bCD27 subsets showed a significant increase in the percent of CD11b^{High}CD27^{Low} and a tendency in the reduction of CD11b^{Low}CD27^{High} cells in NK cells from skin draining lymph nodes of FVB-121 mice compared to WT **(Figure R.19A).** No differences were noted in the CD11bCD27 subsets of FVB-187 mice compared to WT mice **(Figure R.19B)**.

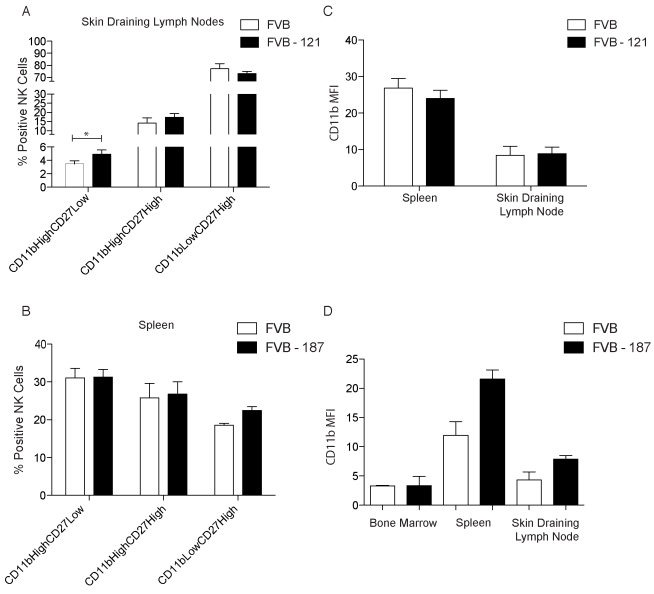

Figure R19 - Analysis of CD11b and CD27 subsets of NK cells from mice expressing systemic levels of Rae-1ε

A,B) Freshly isolated mouse lymphocytes from either skin draining lymph nodes of FVB and FVB-121 mice (a) or spleens of FVB and FVB-187 mice (b) were stained with anti-NKp46 and -CD3 mAb and analyzed for CD11b and CD27 expression. C,D) The median fluorescence intensity of CD11b in NK cells was analyzed for both FVB and FVB-121 (c) mice and FVB and FVB-187 (d) mice. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 3 to 5 mice/group in two independent experiments; *p<0.05).

3.3 Systemic Rae-1 expression correlates with higher IFN-γ production

NK cells from skin draining lymph nodes of FVB-121 and spleens of FVB-187 mice were also analyzed for IFN-γ- production after stimulation with PMA and ionomycin. Constitutive and localized Rae-1ε expression was seen to correlate with increased levels of IFN-γ producing NK cells in FVB-121 mice **(Figure R.20)**. These results indicate that constitutive-localized expression of Rae-1 expression is capable of altering NK cell maturation and IFN-γ secreting capacities.

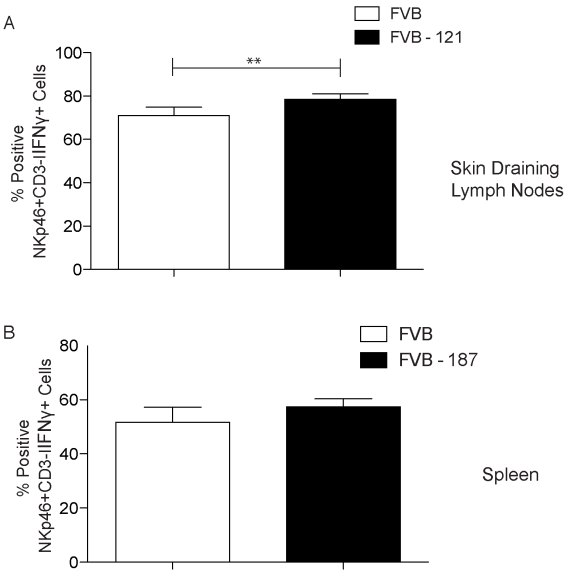

Figure R20 - Production of IFNγ after PMA/ ionomycin stimulation of NK cells from mice expressing systemic levels of Rae-1ε

Lymphocytes isolated either from spleen or skin draining lymph nodes were stimulated with PMA, ionomycin and BFA, and stained with anti-NKp46, -CD45, -CD3, and -IFNγ mAb (4 h). A,B) Percentages of $NKp46^{+}CD3^{-}IFN\gamma^{+}$ cells were calculated from skin draining lymph nodes from FVB and 121 mice (a) and splenocytes from FVB and 187 mice (b) Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 3 to 5 mice/group in two independent experiments; *$p<0.05$).

Part 4 Role of p85β in NK cell signaling

4.1 p85β is expressed in murine NK cells and does not influence the commitment or cellularity of NK cells

In order to define if the PI3K-p85β subunit was expressed in murine NK cells, we analyzed via qRT-PCR the levels of the p85β and of p85α subunits in six day IL-2 activated NK cells isolated from C57BL/6 mice. p85β was detected to the same extent as p85α in NK cells **(Figure R.21 A)**. To define the possible role of p85β in early NK cell development, we analyzed NK cell numbers both in bone marrow and in spleen. Both the percentages and the absolute numbers of NKp46^{+}CD3^{-} cells were found not to vary in neither BM or spleen of p85β$^{-/-}$ mice compared to their p85β$^{+/+}$ (WT) littermates **(Figure R.21 D,E,F,G)**.

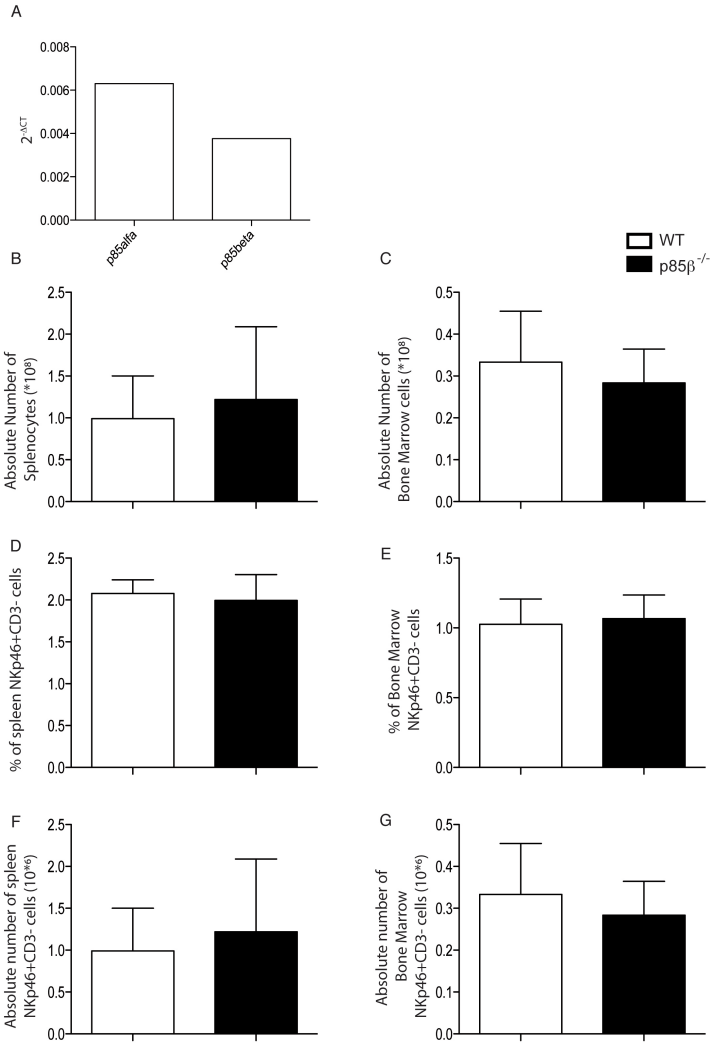

Figure R21 – Phenotypic analysis of NK cell cellularity in p85β deficient mice

A) Total RNA was extracted from spleen derived six day IL-2 activated NK cells from C57BL/6 mice. Expression of the p85α and p85β mRNA was analyzed by qRT-PCR. Normalized quantities to β-actin (mean $2^{-\Delta Ct}$) of p85α and p85β mRNA are shown. B,C) Absolute numbers of spleen (a) and BM (b) cells were calculated from both p85β$^{-/-}$ and WT mice. D,E,F,G) Percentages and absolute numbers of NKp46^{+}CD3^{-} cells were calculated in both spleen (d, f) and BM (e,g). Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 8 mice/group in three independent experiments).

As previously stated, NK cell development can be defined as a five- or six-step process, based on the expression pattern of cell surface markers [9, 29]. The maturity of NK cells can be determined via the expression of various cell markers on its surface, such as CD122, NKG2D, NK1.1, CD49b, CD11b, CD43, CD2, and CD11a. Gated NKp46^{+}CD3^{-} cells were analyzed for expression of these markers. The deletion of the p85β subunit did not affect the expression of any of the aforementioned markers **(Figure R.22)**.

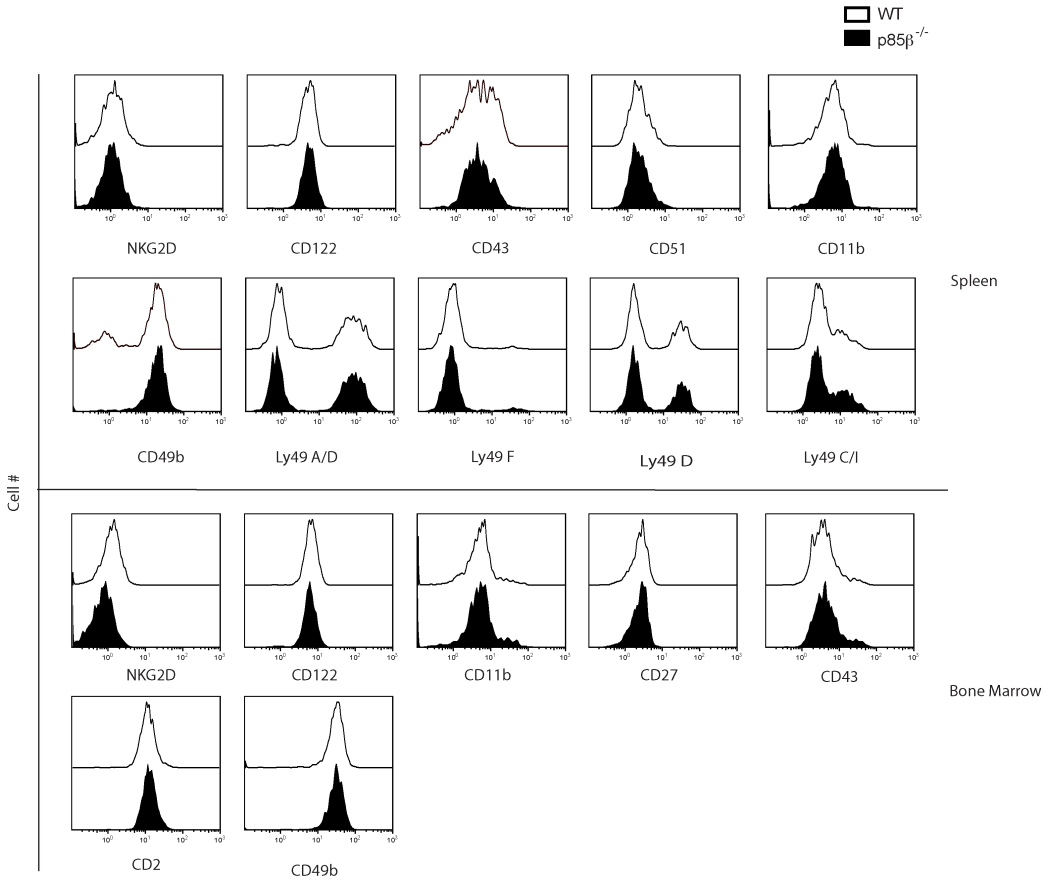

Figure R22 – Analysis of developmental NK markers

Expression of developmental markers in p85β $^{-/-}$ NK cells in spleen and bone marrow. Representative histograms for each marker are shown. Gates were set using unstained or nonspecific isotype mAb controls (not depicted) on NKp46^{+}CD3^{-} cells. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 7-8 mice/group in three independent experiments).

An expression profile of the activating and inhibitory Ly49 receptor repertoire indicated that the expression levels of the Ly49 A, C, D, F, G, or I receptors on NK cells were also comparable between p85β$^{-/-}$ and WT mice **(Figure R.22)**.

NK cell maturation, as previously stated can be further subdivided into another four-stage process, dependent on CD27 and CD11b expression [105]. No differences were seen

between the proportion of the various CD11bCD27 subsets between the p85β$^{-/-}$ and WT mice **(Figure R.23)**.

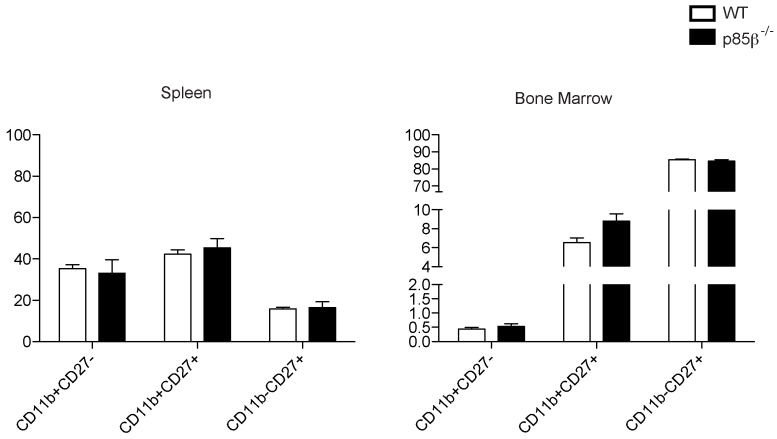

Figure R23 p85β deficiency does not alter CD11bCD27 subsets

Freshly isolated mouse splenocytes and BM cells were stained with anti-NKp46 and -CD3 mAb and analyzed for CD11b and CD27 expression. Percentages were calculated of CD11b^{Low}CD27^{High}, CD11b^{High}CD27^{High}, and CD11b^{High}CD27^{Low} cells for splenocytes and BM cells. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 6 mice/group in three independent experiments)

In order to discard the possibility of p85β being implicated in the initial commitment of NK cells, we decided to look at the generation of NK precursor cells in the BM. NK cell differentiation from NKPs is a complicated process that begins in the BM with the generation of NKP cells, which are CD122^{+}Lin^{-}CD49b^{-}NK1.1^{-}. These cells then follow a developmental pattern that gives rise to immature NK (iNK) cells in which they begin to express some but not all NK-specific developmental markers: CD122^{+}Lin^{-}CD49b^{-}NK1.1^{-} (Stage 1 - NKP) → CD122^{+}Lin^{-}NK1.1^{+}CD49b^{-} (Stage 2) → CD122^{+}Lin^{-}NK1.1^{+}CD49b^{+} (Stage 3) [1, 31, 105].

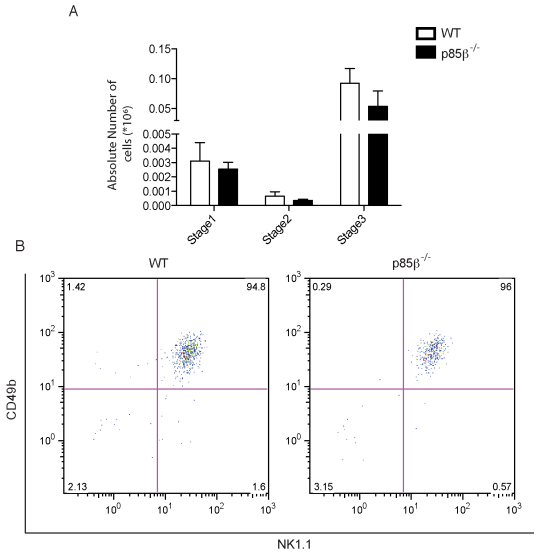

Figure R24 p85β deficiency does not alter NKP or iNK development

A) Absolute numbers were calculated of total Stage1, 2, and 3 iNK cells. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 6 mice/group in three independent experiments) B) Representative flow cytometric density plots show the proportion of CD122^{+}Lin^{-} cells. Plots show proportions of Stage 1 (CD122^{+}Lin^{-}NK1.1^{-}CD49b^{-}), Stage 2 (CD122^{+}Lin^{-}NK1.1^{+}CD49b^{-}) and Stage 3 (CD122^{+}Lin^{-}NK1.1^{+}CD49b^{+}) iNK cell groups in bone marrow

Analysis of the total number of NKP and iNK cells showed no differences between p85β$^{-/-}$ mice and their WT counterparts **(Figure R.24)**.

4.2 p85β does not influence NK cell apoptosis or death

In order to ascertain if NK cells p85β deficiency could be implicated in NK cell death or apoptosis, fresh and 6 day activated BM and spleen NK cells were stained with 7AAD and Annexin V. No differences between the percent of Annexin V^{+}7AAD^{-} and Annexin V^{+}7AAD^{+} were seen neither in fresh or IL-2 activated NK cells between the two groups **(Figure R.25)**.

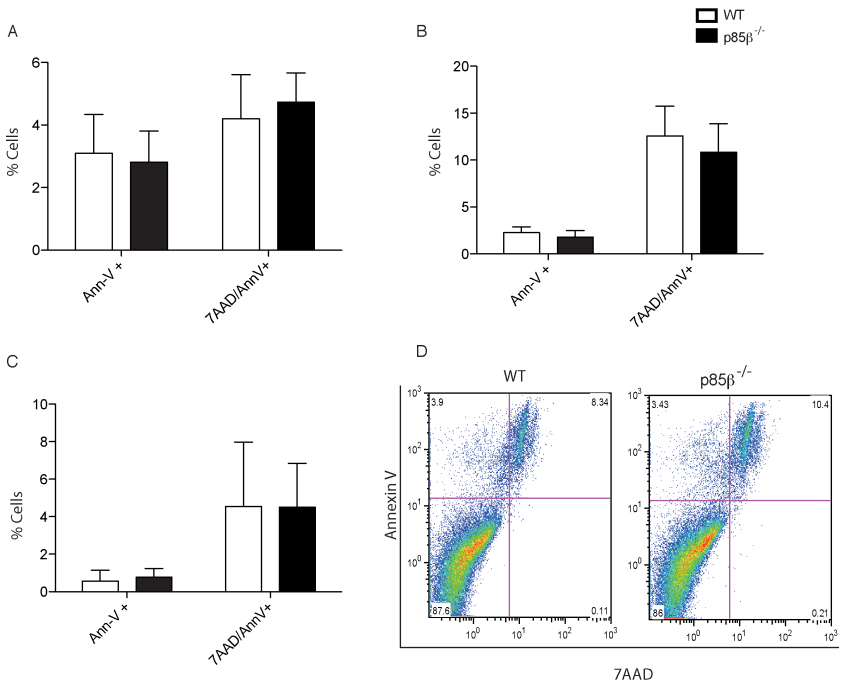

Figure R25 p85β deficiency does not alter NK cell death or apoptosis

A,B,C) Freshly isolated splenocytes (a), bone marrow cells (b), or six day IL-2-activated spleen NK cells (c) were stained with Annexin V and 7-AAD for apoptosis analysis. Gates were set on NKp46^{+}CD3^{-} cells. Percentages of positive cells were determined. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 6 mice/group in three independent experiments). D) Representative flow cytometric density plot shows the proportion of Annexin V^{+}7AAD^{-} and Annexin V $^{+}$7AAD^{+} cells of six day IL-2-activated spleen cells.

4.3 p85β deficiency does not alter IL-2-activated NK cell proliferation

To assess if p85β deficiency was implicated in NK cell proliferation, we performed BrdU proliferation assays on six day IL-2-activated splenic NK cells. Splenic NK cells were cultured for 6 days with IL-2, and then pulsed with BrdU No differences in proliferation were noticed between p85β deficient mice and their WT counterparts **(Figure R.26)**.

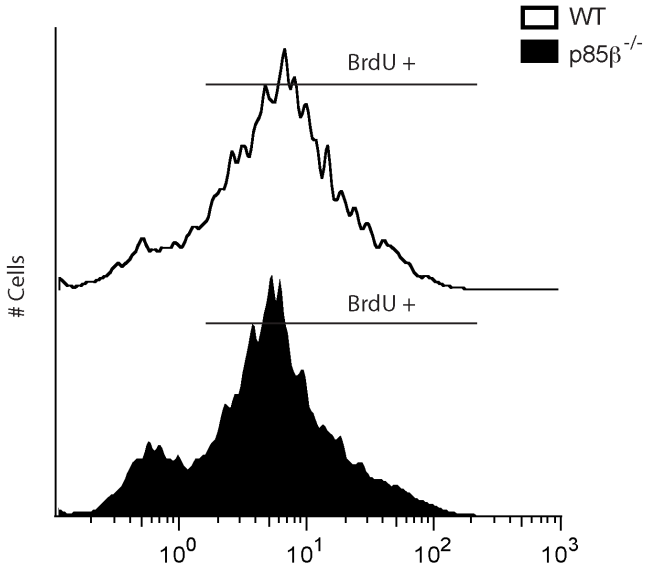

Figure R26 - p85β deficiency does not alter NK cell proliferation

Six-day IL-2-activated splenic NK cells were pulsed with BrdU, and incubated at 37°C for 4 hours. NK cells were then stained and analyzed for $NKp46^{+}CD3^{-}$ proliferation. A representative histogram of one of two independent experiments is shown. Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 2 mice/group in 2 independent experiments)

4.4 p85β deficiency marginally increases NK cell CD107a degranulation but not conjugation

To assess whether BM and spleen NK cells in a SLE environment also have functional defects, we performed conjugation and CD107a degranulation assays using 6-day-cultured IL-2-activated NK cells from $p85\beta^{-/-}$ and WT mice. Conjugation in short intervals was tested against Schneider Cell Line 2 drosophila cells (S2) transfected with ICAM1 (S2-mICAM1) or ICAM1 and MULT-1 (S2-MULT-1/mICAM1) and the YAC-1 cell line. ICAM1 has been shown to be both necessary and sufficient for the correct conjugation of NK cells in humans, and S2 cells have been already shown to be excellent target cells for NK cells after transfection with the appropriate ligands [123]. $p85\beta^{-/-}$ NK cells showed no impaired capacity to conjugate either S2-

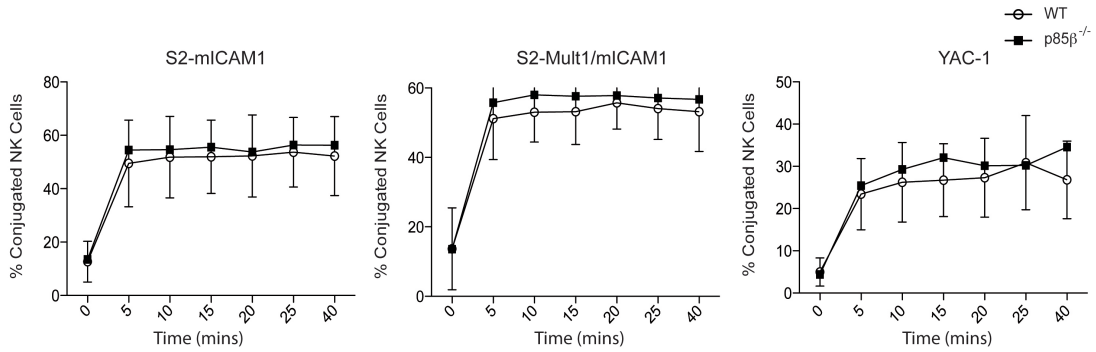

Figure R27 p85β deficiency does not alter NK cell conjugation

Six-day IL-2-activated spleen NK cells were tested in a four-point conjugation assay against S2-mICAM1 (4:1 target effector ratio) , S2-MULT-1/mICAM1 (4:1 target effector ratio) and YAC-1 (2:1 target:effector ratio). Data were analyzed with a two-tailed, unpaired Student's T test (mean ± SD; n = 3 mice/group in three independent experiments)

mICAM1, S2-MULT-1/mICAM1 or YAC-1 cells **(Figure R.27)**. Furthermore, no the co-expression of MULT-1 with mICAM1 did not seem to alter the conjugation capacities of NK cells.

Degranulation of the lysosome-associated membrane protein-1 CD107a (LAMP-1) is upregulated on the NK cell surface following stimulation with target cells able to induce a cytolytic response, and occurs after NK cells have successfully conjugated to their target cells. To determine whether or not p85β deficiency could be implicated in CD107a degranulation, 6-day-cultured IL-2-activated NK cells from p85β$^{-/-}$ and WT mice were stimulated against the YAC-1 (NKG2D ligand expressing) and CHO (which express a homologue of H2-Dd) target cells. A tendency in the increase of CD107a degranulating p85β$^{-/-}$ NK cells after stimulation with both CHO and YAC-1 cells was noted compared to WT NK cells **(Figure R.28).**

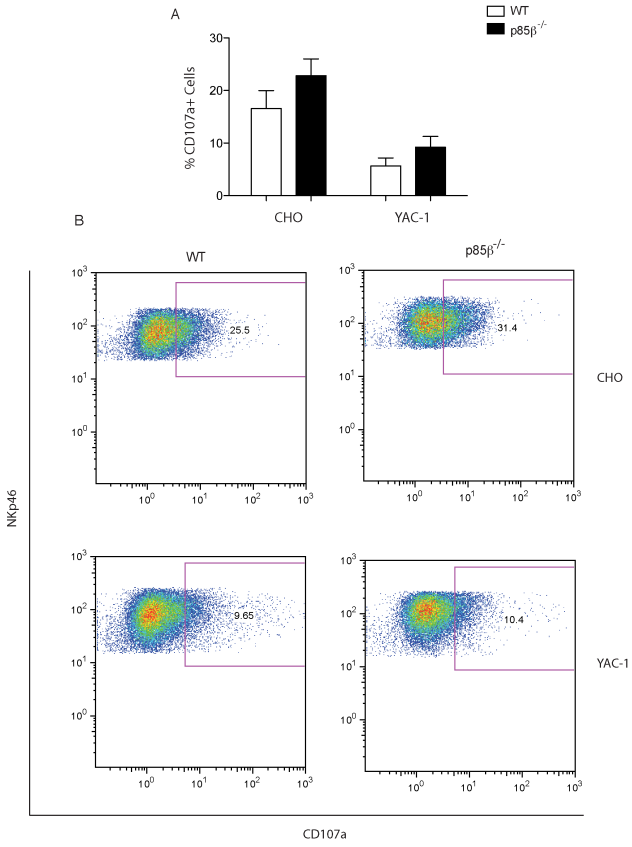

Figure R28 Analysis of CD107a secretion in p85β deficient mice

A,B) Six-day IL-15-activated spleen or BM NK cells were tested against the CHO and YAC-1 target cell lines at a 2:1 target:effector ratio in a 4 h CD107a degranulation assay. Representative stainings are shown (b); data were analyzed with a two-tailed, unpaired Student's T test.

4.5 p85β deficiency augments NK cell cytotoxicity

In order to evaluate if p85β deficiency influenced NK cell cytotoxicity, we performed NK cell cytotoxicity assays with 6-day-cultured IL-2-activated NK cells. As target cells we used the NKG2D ligand expressing cell line, YAC-1, and S2 cells transfected with the MULT-1 NKG2D ligand and murine ICAM1 (S2-mICAM1/MULT-1). IL-2-activated p85β$^{-/-}$ NK cells seemed to show marginally enhanced cytotoxicity towards both target cells compared to their WT

counterparts **(Figure R.29).**

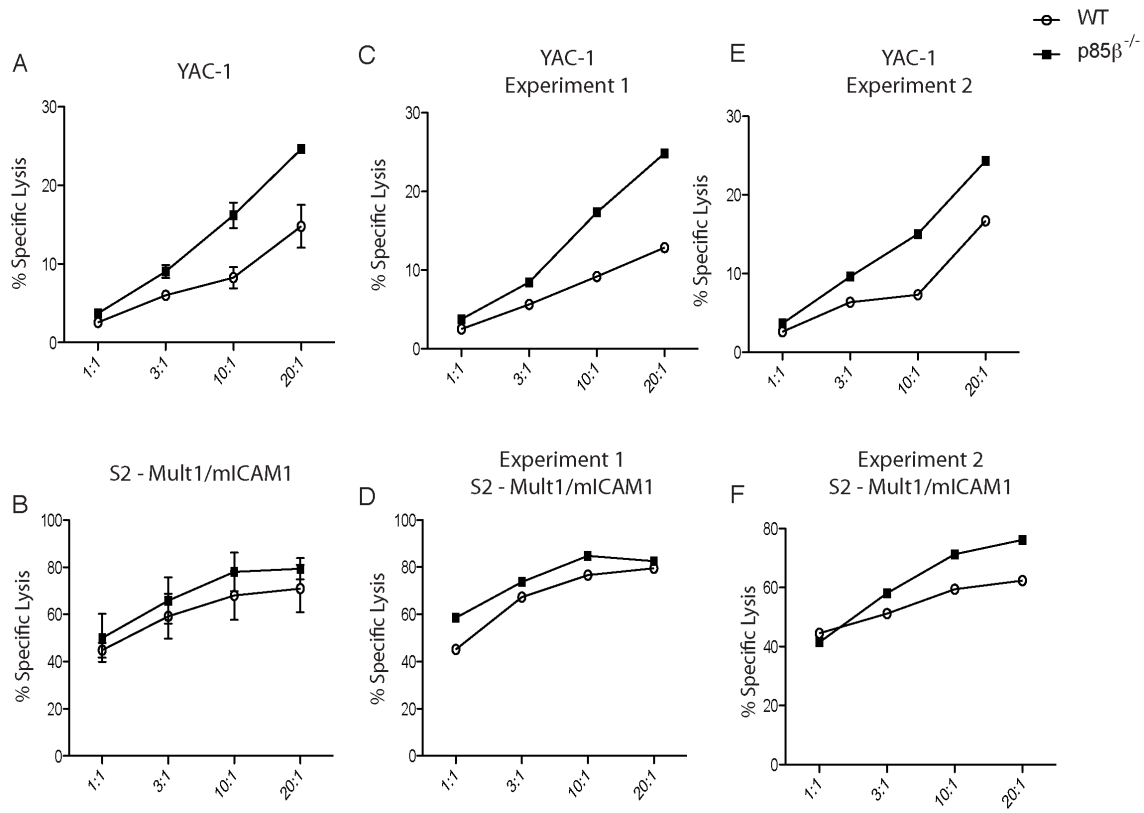

Figure R29 IL-2-activated p85β-/- NK cell cytotoxicity is moderately increased

IL-2-activated splenic NK cells were tested against PKH67 labeled target cells at the indicated effector/target ratios. Cytotoxicity was tested against the YAC-1 and S2-MULT-1/mICAM1 cells. Shown are the means of three independent experiments for S2-MULT-1/mICAM1 and 2 independent experiments for YAC-1 (a,b) , and two representative individual experiments with each target cell (c-f). Data were analyzed with a two-tailed, unpaired Student's T test

4.6 p85β deficiency increases NKG2D activated cytokine generation

NK cells are known producers of various pro-inflammatory cytokines, of which one of the most important is IFNγ. Therefore, we tested NKp46^{+}CD3^{-} NK cells for IFNγ generation. Six day spleen derived IL-2 activated NK cells were stimulated with anti-NKG2D and anti-NK1.1 antibodies. mAb-stimulated NK cells were seen to produce large amounts of IFNγ upon stimulation, yet p85β$^{-/-}$ cells showed increased IFNγ production when stimulated compared to WT NK cells. **(Figure R.30A)**.

Via luminex, we also checked the levels of other typical NK cell cytokines. Albeit having performed only one experiment with two samples, we noticed an increase in the levels of IP10, IL10, TNF-α, IL-6, and RANTES in p85β deficient IL-2 –activated NK cells compared to WT cells after stimulation with both NK1.1 and NKG2D **(Figure R.30B)**.

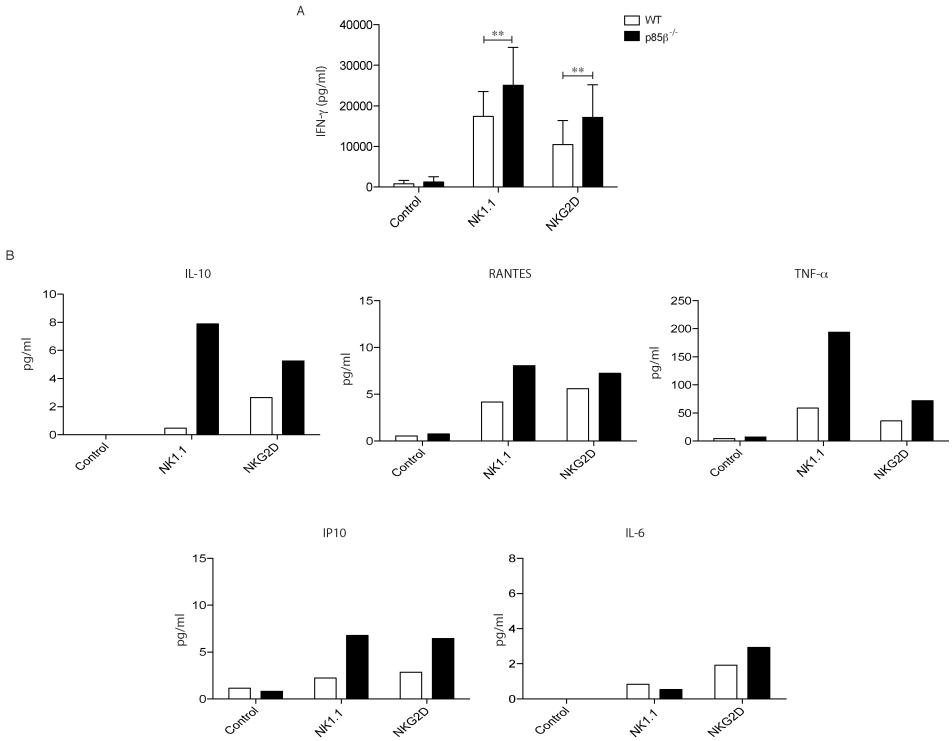

Figure R30 IL-2-activated p85β-/- NK cell cytokine generation is increased

A) 2*10^5 IL-2-activated splenic NKp46$^+$CD3$^-$ cells per well were activated with plate-bound anti-NKG2D (A10) or anti-NK1.1 (PK136 mAbs) and the supernatants were tested for IFN-γ via ELISA (mean ± SD; n = 5 mice/group in 5 independent experiments; **p<0.01). Data were analyzed with a two-tailed, unpaired Student's T test B) 2*10^5 IL-2-activated splenic NKp46$^+$CD3$^-$ cells per well were activated with plate-bound anti-NKG2D (A10) or anti-NK1.1 (PK136 mAbs) and the supernatants were tested for the indicated cytokines and chemokines in a Luminex assay. (n = 2 mice/group in 1 experiment).

4.7 p85β deficient NK cells show impaired NKG2D downregulation

Recent evidence has suggested that PI3K kinases might play a role in the internalization of receptors which include the PI3K p85 binding motif [124]. To evaluate the possible effect p85β could have on the regulation of the NKG2D activating receptor, we decided to test for NKG2D downregulation. This was achieved by stimulating freshly isolated NK cells with the NKG2D activating antibody (clone A10) at various time points, and then by analyzing cell surface expression of NKG2D with the use of the CX5 antibody which stains for another epitope of NKG2D. Our analysis show a decrease in the internalization of the NKG2D receptor in p85β$^{-/-}$ NK cells compared to WT NK cells **(Figure R.31)**.

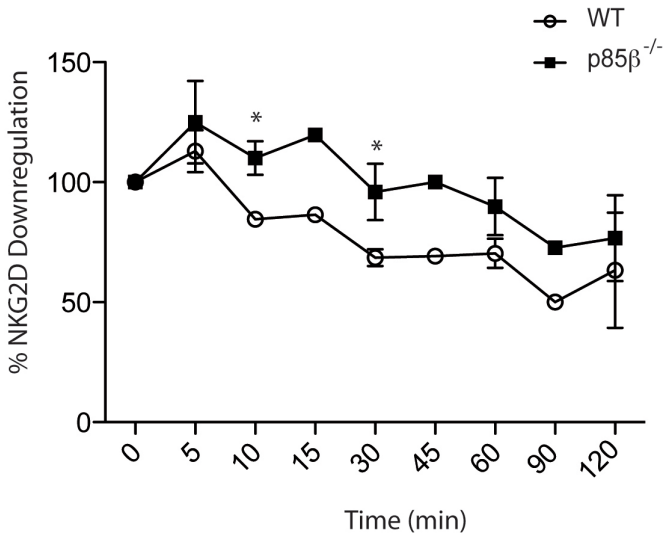

Figure R31 p85β deficiency correlates with defects in NKG2D receptor internalization

p85β$^{-/-}$ and WT NK cells were stimulated with an activating NKG2D mAb (A10) for indicated time points and KIT receptor internalization was studied by staining the cells with PE-conjugated anti-NKG2D antibody (CX5) followed by flow cytometric analysis. (mean ± SD; n = 3 mice/group in three independent experiments; *p<0,05) Data were analyzed with a two-tailed, unpaired Student's T test

Discussion

In order to be accepted as a model, a theory must seem superior to rival theories, but does not need– nor will it ever - explain all of the events it will encounter

Thomas S. Kuhn

Science may be described as the art of systematic over-simplification — the art of discerning what we may with advantage omit.

Karl Popper

Possible role of NK cells in SLE

Whereas the MRL mouse strain has long been used to study SLE, little attention has been paid to the role of NK cells in this model. To date, the only information on NK cells in the periphery of human SLE patients indicates reduced expression and number of $CD122^+$ NK cells, a reduction in total NK cell number and in their cytotoxicity [71, 72]. Proliferation, cytotoxicity and differentiation defects are reported for NK cells derived from haematopoietic BM cells from SLE patients, but not from fresh BM samples [71]. The $MRL/MpJl^{pr}$ is of great use to the study of SLE due to the rapid onset of SLE-like symptoms, but its accelerated phenotype is due mainly to the recessive autosomal mutation which alters the transcription of the Fas receptor [101]. Indeed, even though this model mimics many aspects found in SLE patients and that defective Fas signaling has been correlated with the development of autoimmune lymphoproliferative syndrome (ALPS), which shares many symptoms with SLE, the extreme autoimmune manifestations that these mice exhibit, such as the immense accumulation of double negative and $B220^+$ T cells are neither typical nor common in all SLE patients [125]. However, it is known that the lpr mutation by itself is not capable of resulting in glomerular nephritis, and that Fas sufficient MRL/MpJ mice have background genes which are capable of promoting and developing SLE-like symptoms, in a more "natural", albeit slower manner compared to MRL/MpJ^{lpr} mice [101, 126]. Nonetheless, only the MRL/MpJ^{lpr} mouse strain has been slightly used to study the role of NK cells in SLE, and no information to date has been published regarding the role of NK cells in diseased MRL/MpJ mice.

To our knowledge, the phenotype of NK cells in a SLE/SLE-like environment had not been extensively characterized. Here we confirm previous data indicating a reduction in the percentage of NK cells in diseased LPR mice and show a similar reduction in diseased, 1-year-old MPJ mice, indicating their usefulness for the study of NK cells in SLE [72]. Comparison of percentages and absolute numbers of $NKp46^+CD3^-$ cells in diseased and healthy LPR and MPJ mouse BM suggested that the reduction observed in the periphery in diseased mice is not due to defective NK cell production in BM

We analyzed cell surface expression of NK markers in SLE-like mouse BM and spleen to determine the influence of microenvironmental/organ-specific differences on NK cell phenotype. $NKp46^+CD3^-$ cells in diseased mouse spleen showed downregulation of mature NK cell markers, which was more notable than in BM. There were no differences in BM CD11b, CD43, CD122 or CD2 expression levels between healthy and diseased MPJ and LPR mice. In diseased mouse spleen, NK cell surface levels of CD43, CD49b, CD122, CD2 and CD11b were significantly downregulated. We thus postulated that in diseased mice, the SLE-like microenvironment already affects NK cell maturation in BM, although to a much lesser extent than in spleen. Several reports show a non-mature functional phenotype

in peripheral NK cells from SLE patients and from diseased LPR mice, characterized by reduced CD122 expression and cytotoxicity [71, 76, 77]. We found that this non-mature functional phenotype correlates with decreased expression of various mature NK cell markers. High CD11b, CD43, CD49b and CD122 levels are markers of functional, mature NK cells; their reduction in diseased LPR and MPJ mice thus indicated a probable increase in less-mature NK cells.

To determine whether expression of these defining markers for NK cell maturation decreased after acquisition of a fully mature state or if the NK cells suffered developmental defects, we analyzed NK cell precursor numbers in BM and in spleen. This four-stage process is far better understood in mice than in humans (NK1.1, a defining marker for NK cell development, was not considered as it is not expressed in the MRL strain). As previously stated, NKP cells have been found in various organs outside the BM, it is thought that they arise in BM, travel to other organs and develop fully in situ. We thus proposed that in diseased mice, NK cells develop differently in spleen than in BM. In accordance with our data, we found no differences in total NKP or iNK cell numbers in any of the four developmental stages in BM. In the spleen of diseased mice, we observed accumulation of iNK and NKP cells in Stages 1 2, and 3; this led us to postulate that NK cells do not develop correctly in diseased mouse periphery. NK cells in diseased patients are thought to suffer impaired maturation and differentiation, and indeed, NK cell activation and maturation is influenced by microenvironmental factors such as the cytokine milieu or marrow stromal cells [77, 127, 128].

These data correlated with our analysis of CD11bCD27 NK cell subsets in spleens from SLE-like mice. Accumulation of $CD11b^{Low}CD27^{High}$ and a decrease in the percentage of $CD11b^{High}CD27^{Low}$ NK cells is further evidence that, in diseased mouse spleen, NK cells do not develop a completely mature phenotype. Furthermore, this increase in the $CD11b^{Low}CD27^{High}$ could possibly mimic the $CD56^{Bright}$ population which has been found to be increased in SLE patients with an active disease, and which are known to represent the same immature NK cell subtype in humans [129, 130].

To determine whether decreased expression of these mature NK cell markers correlated with functional deficiencies, we performed conjugation and CD107a degranulation assays using 6-day-cultured IL-15-activated NK cells from BM and spleen. We used the YAC-1 cell line for conjugation assays, as it expresses a variety of cell surface receptors involved in NK cell conjugation, including ICAM1, ICAM2, and CD48. Preliminary data we collected showed that CHO and RMA/S cells did not conjugate well at short times (not shown). The assays indicated no differences in the conjugation capacity of activated BM NK cells from diseased LPR and healthy MPJ mice. Activated spleen NK cells from diseased LPR mice nonetheless showed decreased ability to form target-effector cell conjugates at longer times; this defect could be due to the decrease in CD2-expressing NK cells in these mice, as the CD2 ligand CD48 has a decisive role in strengthening formation of NK cell conjugates [123].

Studies to date have focused on measuring the cytotoxicity of NK cells from SLE patients and SLE-like murine models. Since CD107a degranulation correlates with NK cell-mediated cytotoxicity, we used this assay as an alternative to measure NK cell cytotoxicity. After stimulation with various target cell lines, IL-15-activated spleen and BM NK cells from diseased LPR mice had fewer CD107a-expressing cells than healthy MPJ mice. These data confirm reports of reduced NK cell cytotoxicity in spleen and peripheral blood, although here we used freshly-extracted, activated BM NK cells directly in a cytotoxic assay [71, 72]. These data might slightly contradict a previous study in which IL-2-activated human NK cells from peripheral blood of patients with active SLE showed no differences in degranulation compared to healthy controls [77]; these differences might be due to the use of distinct cytokines used for NK cell activation and/or target cell lines. Another study showed reduced cytotoxicity of human haematopoietic stem cells differentiated in vitro to NK cells [71]; our results add to the data from this report, as we used IL-15-activated NK cells extracted directly from BM. The CD107a degranulation defect found both in BM and periphery allows us to postulate that the SLE microenvironment in diseased mice is already able to reduce the cytotoxic capacity of mature NK cells in BM. Whereas cytometric analyses of NK cell surface expression markers appear to indicate that NK cells from diseased mice show a more pronounced "immature" phenotype in spleen than in BM, BM NK cells might already be in a context that influences their functional capacity.

There is little information regarding the phenotypic and functional aspects of NK cells in SLE target organs. To date, only three studies have shed some light on this aspect. Two studies reported that the phenotype of liver and lung NK cells from diseased MRL/lpr mice was more cytotoxic than that of healthy MRL/MpJ or MRL/lpr mice [131, 132]. In another, PMA- and ionomycin-stimulated NK cells from diseased MRL/lpr mice produced more cytotoxic granules compared to prediseased MRL/lpr or healthy MRL/mpj mice [72]. These data imply that the phenotype of NK cells in target organs might be reversed compared to NK cells in the periphery. Our first step was to analyze via flow cytometry the lymphocyte populations present in healthy and SLE-like diseased kidneys. Our analysis revealed an increase in the total number of CD45 lymphocytes in diseased kidneys compared to their respective controls. Furthermore, as has been already noted in other non-lymphoid organs, the percent of NK cells in healthy kidneys accounts for around ≈10% of the lymphocyte population. The percent of NK cells, as in the periphery, was seen to diminish in diseased kidneys compared to healthy ones, even though the absolute numbers of NK cells did not vary, except for an increase in the diseased LPR mice.

Our cytometric staining of the NK cells from lymphocytes derived from healthy and diseased mouse kidneys showed that NK cells in diseased kidneys did not downregulate markers that were downregulated in BM and spleen. Moreover, NK cells from diseased mice suffering

severe nephritic lupus had increased CD11b levels compared to healthy mice, suggesting a higher activation state of NK cells in inflamed kidneys. This increased CD11b expression in diseased kidney NK cells correlated with an increase in the percentage of $CD11b^{High}CD27^{Low}$ cells and a decrease in the percentage of $CD11b^{Low}CD27^{High}$, another indication of a more mature phenotype in NK cells in nephritic kidneys compared to peripheral NK cells. This CD11b increase correlated with a larger percentage of kidney-infiltrating NK cells, which respond to PMA and ionomycin stimulation and thus produce higher IFN-γ levels. We found significant differences between the percentage of IFN-γ-producing cells in diseased and healthy mice specifically in the $CD11b^{High}CD27^{Low}$ population, indicating that these more mature, infiltrating NK cells are responsible for the increased IFN-γ production. While the literature to date seems to support the idea that $CD27^{High}$ cells are the main effector cells and IFN-γ producers [36], it might be possible that these kidney infiltrating NK cells are part of a distinct NK cell subtype. These kidney infiltrating NK cells might have a more pronounced "inflammatory-helper" role, rather than acting as direct effectors of cytotoxicity. Unfortunately, since we were incapable of isolating and culturing kidney infiltrating NK cells, we were not capable of testing the cytotoxic capacities of these $CD11b^{High}CD27^{Low}$ kidney cells against NK cell targets.

STAT5 plays a crucial part in NK cell signaling, given its role in the development and survival of NK cells, and in transducing and activating a myriad of biological processes after NK cell stimulation with cytokines such as IL-2, IL-15, and IL-21 [110, 133]. To determine whether the phenotypic differences in the various organs correlated with deficient STAT5 phosphorylation, we incubated freshly isolated NK cells (mature $CD45^{+}NKp46^{+}CD3^{-}$ cells) with IL-15. The results showed no significant differences between the phosphorylation levels of STAT5 in NK cells from spleen or BM. On the other hand, NK cells from diseased kidneys were seen to significantly phosphorylate more STAT5 compared to NK cells from healthy and prediseased kidneys. Moreover, the fact that kidney NK cells phosphorylated more STAT5 in general compared to NK cells from BM or spleen suggests that kidney resident/infiltrating NK cells have a generalized more active/mature state, which is hard to explain only based on the CD11b and CD27 subsets.

In order to identify possible mechanisms that could be implicated in the differences in NK cell maturation which we report between diseased and healthy kidneys, we analyzed the levels of IL-15 and IL-12 in the kidneys of all four mouse groups. Our analysis revealed what seemed to be a correlation between higher levels of IL-15 in diseased kidneys compared to healthy kidneys. IL-15, being one of the main players in NK cell development, could thus be one of the likely candidates implicated in the more mature phenotype which we report in the kidneys of diseased SLE-like mice. Furthermore, the increase of IL-12 in prediseased LPR kidneys

might mimic data found in patients, in which increases of serum IL-12 was found right before disease flares [134]. Thus IL-12 levels might increase and participate in NK cell activation in the stages which immediately proceed an active disease state in diseased kidneys. Similarly, the increase of CX3CL1 in the kidneys of prediseased LPR mice, might instead be the cause for the infiltration, and thus higher numbers of NK cells, typical only to the LPR, but not MPJ SLE-like model. A possible explanation for our detected increase of CX3CL1 and IL-12 in prediseased LPR kidneys but not healthy MPJ kidneys would probably be due to the time point in question, seeing as how healthy MPJ kidneys would still be months away from developing any sort of inflammatory state.

We here report Rae1 and MULT-1 expression specifically in glomeruli of the MRL mouse strain. As this strain develops lupus-like disease independently of the Fas mutation in the MRL/MpJ^{lpr} mice, it appears that the genetic background of these mice predisposes to late-onset SLE-like symptoms. In kidney, expression of these ligands is specific to the glomeruli, which allows us to postulate that this predisposed SLE background is the cause of NKG2D ligand expression, not present in C57BL/6 or BALB/c control strains. We also found a correlation between heightened Rae-1 expression in the glomeruli of diseased mice compared to healthy controls. The glomeruli of mice in an active disease state might thus be subject to a broader "stress-induced" environment, which could be responsible for increased expression of this NKG2D ligand. We also report Rae-1 expression in the glomeruli of diseased NZBxNZW(F1) mice, another glomerulonephritis model. This confirms expression of detectable NKG2D ligand levels in glomerulonephritic kidneys of three SLE-like models.

To see if the same pattern of NKG2D ligand expression was also seen in SLE patients, we analyzed kidney biopsies of patients with an active lupus nephritic state for the expression of the human NKG2D ligands MICA and ULBP1. Our immunohistochemical stainings only showed one patient which showed a very light staining for ULBP1, but four patients which showed mild to intense staining of MICA in the glomerular and peritubular endothelium. Even though we were not capable of reproducing such a specific glomerular stain of NKG2D ligands, such as what was noted in our SLE-like mouse models, the pattern of expression of MICA in patients with active SLE resembles what has been previously reported in kidney sections from patients suffering from Wegener's granulomatosis [135]. Wegener's granulomatosis is an inflammatory disease of unknown origin, and whose most life-threatening symptom is its necrotizing glomerulonephritis. Patients with an active state of Wegener's granulomatosis showed both increased levels circulating MICA^{+} cells in the blood and positive expression of MICA in both the glomerular and peritubular epithelium in the kidneys [135]. Thus, the expression of MICA, even though not glomerular specific, seems to be specifically upregulated during the active

stages of inflammatory-autoimmune kidney manifestations. This together with reports that link MICA polymorphism to the pathogenesis of SLE via the possible modulation of $CD4^+$, NK, and/or monocytes is an interesting link to the possible effects of MICA expression in the kidneys of patients with active SLE [78, 136, 137].

Based on the analysis of kidney infiltration levels of NKG2D-expressing cells (NK, CD4 and CD8 cells), we report the participation of these cytotoxic cells in glomerulonephritis. It has already been reported that NK, and other NKG2D-expressing cells can accumulate in tumors expressing NKG2D ligands [138, 139]. Using immunohistochemical techniques, we observed significantly higher numbers of glomeruli with cytotoxic cellular infiltrates in diseased mouse kidneys compared to pre-diseased and healthy controls. This result was not reproduced in cytometric analysis, which only indicated an increase in total numbers of $CD4^+$, $CD8^+$ and NK cells only in diseased LPR mouse kidney. During glomerulonephritis, NK cells might thus have a qualitative rather than a quantitative role, as there appears to be no notable difference in total NK cell numbers between diseased MPJ mice and the control groups. Notwithstanding, this result contrasts with a previous study in which larger numbers of NK cells were found in kidneys of pre-diseased compared to diseased LPR mice [72]. Whereas the discrepancies with our results could be attributed to the distinct techniques and cell markers used for NK cell screening (we use NKp46, CD45, and a live/dead cell discrimination marker), the previous report was limited to total cell count in the whole kidney, and did not consider the percentage of NK cells that participate in the glomerulonephritic process. Regardless of whether or not total NK cell numbers increase in diseased or pre-diseased kidneys, our data show a correlation between infiltration of mouse glomeruli and an active disease state.

In order to evaluate if the increased glomerular expression of NKG2D ligands could be a possible cause for the more NK cell phenotypes we noticed in the kidney of diseased SLE-like mice, we used the FVB-121 and FVB-187 mouse models, which constitutively express an isoform of the Rae-1 ligand (Rae-1ε) either locally in the epidermis, or systemically. While the analysis of the specific effects of constitutive Rae-1 expression in the FVB-187 model is challenging, due to the fact that Rae-1 is expressed systemically (even on the NK cells themselves) [95], the FVB-121 model serves as a more localized "physiological" form of increased NKG2D ligand expression, which we believe more accurately mimics what occurs in the kidneys of the SLE-like mice (localized expression of Rae-1). Veritably, the localized expression of Rae-1 correlated with higher levels of mature $CD11b^{High}CD27^{Low}$ NK cells and with an NK specific higher IFN-γ production compared to the FVB-187 mice, which showed the same tendencies, but to a lesser extent. The maturation profile of NK cells in the skin draining lymph nodes of FVB-121 mice thus closely resembles that of kidney infiltrating NK cells in diseased SLE-like

mice. Thus, if Rae-1, or NKG2D ligand expression in general is capable of influencing NK cell maturation and/or functional capacities, it still remains to be discovered what threshold of expression these ligands must have in order to exert this effect. Indeed, if healthy, SLE-prone mice already express low levels of Rae-1, what is the Rae-1 expression threshold that has to be passed in order for it to be capable of regulating NK cells?

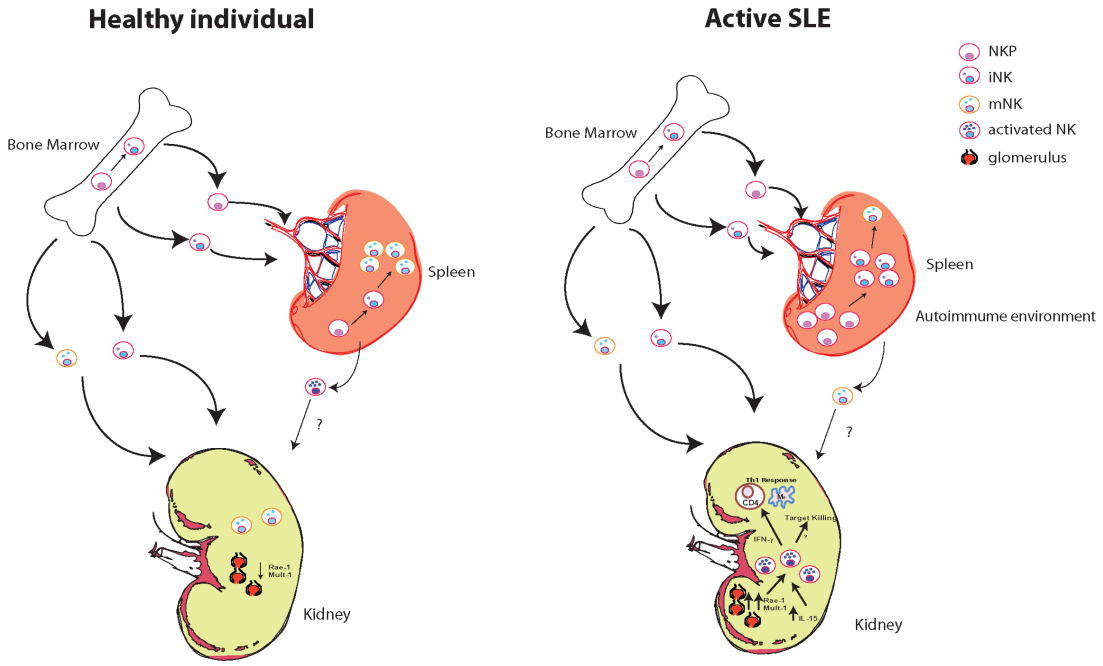

Figure D1 Model for altered NK cell phenotype during active SLE

In this model, we propose that the more activated NK cell phenotype in the kidneys of mice who are in an active SLE-like disease state is possibly due to both the increase expression levels of Rae-1 in the glomeruli and of total IL-15R/IL-15 levels in the kidneys. These two factors, could explain the increase in the levels of mature NK cells, and in their increased secretion of IFN-γ. In the spleen, yet unknown factors, either genetic or environmental, in the accumulation of phenotypic and functionally immature NK cells.

In conclusion, we believe that these results provide evidence for microenvironmental, organ-specific development and regulation of NK cells, which could clarify previous findings that showed impaired terminal differentiation in peripheral NK cells from human SLE patients. Our data emphasize a larger role for NK cells in SLE target organs than previously thought, and contradict the general postulation that NK cells found in PBMCs of patients can accurately reflect the phenotypic and/or functional properties of NK cells found in target organs of SLE. It must also be noted, that extreme caution must be used in the interpretation of NK cell data coming from PBMCs of SLE patients, since prednisone and mycophenolic acid are among the most common treatments for SLE, and both drugs are able to downmodulate NK cell function, cytotoxicity, and proliferation [140-143]. The difficulties of being capable of applying this

knowledge to the clinical world are obvious, even though we believe that these results indicate that extreme care must be taken even in clinical evaluations to disregard NK cells as non-players in SLE. Indeed our results show that NK cells infiltrate the glomeruli of glomerulonephritic SLE-like mice, which is concomitant with an increase in localized glomerular expression of NKG2D ligands. Further work is needed to determine the potential of NK cells, the NKG2D receptor and its ligands in the development of lupus-based glomerulonephritis, to identify potential therapeutic targets.

p85β function in murine NK cells

The role of the p85 binding unit in DAP10 mediated signaling in NK cells has long been validated as being of crucial importance in NKG2D mediated cytotoxicity [83-85]. However, the specific role the p85β subunit plays in NKG2D signaling has not yet been established. By analyzing NK cell levels, and expression of developmental markers both in spleen and BM, we found that p85β deficiency does not seem to be correlated with any defects in NK cell development or maintenance. Surprisingly, we noticed that p85β deficiency caused NK cells to exhibit an activated phenotype. Both via natural (via the NKG2D or Ly49G pathways) and antibody mediated activation (with NK1.1 and NKG2D), a subtle, but constant increase in the cytotoxic, degranulating, and cytokine secreting capacities of NK cells was noted. The increase in cytokine generation is of special interest given that previous studies implicated the DAP12 via the Syk/ZAP70 pathway to be responsible for NKG2D mediated cytokine production [82, 144, 145]. This would imply a new mechanism through which the absence of the p85β subunit is capable of leaving NK cells more prone to a constant activatory state.

This activated phenotype which we have noticed in the p85β deficient mice is moreover completely opposite to two previous reports which have used p85α deficient mice [146, 147]. In one study, p85α only deficient mice showed no developmental defects, even though their functionality was not tested [147]. In another study, the last five exons of the PI3KR1 gene (which can transcribe either for p85α or the p50α or the p55α PI3K regulatory subunits) were disrupted, giving rise to p85α/p50α/p55α deficient mice [146]. These mice showed reduced NK cell numbers both in BM and liver, and this seemed to be attributed to a reduction in the numbers of $CD3^-CD122^+NK1.1^-CD49b^-$ NK cell progenitors. The remaining NK cells seemed to have reduced expression of the Ly49 repertoire (Ly49A, Ly49G2, and Ly49D subsets) and of the mature NK cell marker CD43. Furthermore, NK cells from these to p85α/p50α/p55α mice were also seen to have reduced cytotoxic capabilities against RMA/S and YAC-1 cells and also had impaired cytokine production after stimulation with NK1.1 and NKG2D mAbs. The authors of this paper discuss that the reason for their deletion of the p50α and p55α isoforms was to inhibit any participation of these isoforms in p85α signaling. If the NK cell phenotype in these mice is thus due only to the lack of the p85α isoform, this would mean that the p85β isoform plays a radically different role in the NKG2D/DAP10 signaling in NK cells. However, the grave differences between the p85α only deficient mice and these p85α/p50α/p55α mice seem to indicate that this phenotype might be influenced by the lack of the p50α and/or p55α subunits in conjunction with the lack of p85α.

The implication of the p85β subunit in the internalization of the NKG2D would imply a novel role for PI3K kinases in NKG2D signaling. The impaired internalization of the NKG2D

receptor could also explain the activated phenotype p85β NK cells exhibit, especially after NKG2D activation. A plausible model would be that after activation, impaired NKG2D downregulation would permit NK cells to signal actively via the NKG2D receptor for a prolonged period of time. Since c-CBL levels were seen The exact mechanism through which p85β might mediate NKG2D receptor internalization is yet to be discovered, however this deficiency is likely to contribute to this enhanced NK cell activatory status. In T-cells, p85β's role in TCR downregulation was mediated via p85β recruitment of CBL and c-CBL (proteins involved in T-cell signaling and protein ubiquitination), which participate in TCR degradation [93]. A recent study has shown that the downregulation of c-CBL in human NK cells increases NK cell responsiveness and activation after stimulation via the NKG2D receptor [148]. Thus, seeing how it is known how c-CBL/p85 interactions are crucial in TCR downregulation, it might be interesting to speculate that this might be one of the possible mechanisms through which p85β is involved in NKG2D receptor internalization after stimulation.

While much attention has been paid to the kinase-dependent role of the PI3K family, new found evidence does suggest a role for the PI3K kinases in receptor internalization, possibly as scaffold proteins [124]. The role PI3Ks play in receptor internalization have not been extensively studied, even though it has been noticed that some PI3K isoforms, such as p110γ and p110β play a role in the internalization of either G protein-coupled receptors, such as the β-adrenergic receptors, or the activated human platelet derived growth factor receptor [149, 150]. Furthermore, two previous reports have also noted a role for the p85β isoform in receptor internalization, as p85β deficiency leads to defective KIT receptor down-regulation in bone marrow derived mast cells and to defective TCR downregulation in peripheral CD8 T cells stimulated with CD3 and CD28 mAbs [93, 94]. Moreover, this increase in NK cell activity would also be consistent with a previous report in which p85β deficient T cells showed an increase in proliferation after stimulation with anti-CD3 and IL-2 [151]. In order to evaluate if this enhanced activation has also a biological significance in vivo we are currently performing tumor xenograft experiments using both NKG2D ligand specific and unspecific tumor cells on p85β mice.

Considering its role in inhibiting the correct internalization of NKG2D, p85β might be in perspective a novel drug target for certain types of tumors which are capable of reducing surface levels of NKG2D via the secretion of soluble NKG2D ligands [152]. The importance of the NKG2D receptor in tumor clearance has already been well documented, and thus targeting the p85β subunit might be a possible therapy in inhibiting a well-documented tumor-evasion method [153, 154].

Conclusions

Our studies in the possible role of NK cells in the pathogenesis of NK cells using murine SLE models demonstrate the following:

1. The diseased MRL/MpJ model has reduced proportions and absolute numbers of NK cells in the spleen compared to their healthy counterparts, effectively mimicking NK cell lymphopenia found in SLE patients with an active disease.

2. The MRL SLE-like diseased MRL/MpJ and MRL/MpJ^{lpr} show a decrease in the expression level of mature NK cell markers to a more grave extent in spleen than in bone marrow

3. The MRL SLE-like diseased MRL/MpJ and MRL/MpJ^{lpr} mice show accumulation of immature NK cells in the periphery but not in the BM. An increase in the number of Natural Killer precursor and immature CD11b^{Low}CD27^{High} NK cells was found in the spleen and not bone marrow of diseased mice. IL-15 activated NK cells from diseased MRL/MpJlpr mice show impaired CD107a and conjugation capacities.

4. The majority of kidney infiltrating NK are composed of the mature CD11b^{High}CD27^{Low} population. Kidney NK cells from SLE-like diseased MRL/MpJ and MRL/MpJ^{lpr} mice have a more activated phenotype compared to healthy and predisease controls. There are more CD11b^{High}CD27^{Low} cells in diseased kidneys, and these produce more IFN-γ and phosphorylate more STAT5 than NK cells from the periphery. The increased levels of IL-15R/IL-15 complex in kidneys of diseased mice could explain the increased maturation state of NK cells in this organ.

5. Rae-1 and Mult-1 NKG2D receptor ligands are expressed specifically in the glomeruli of mice that will develop SLE-like symptoms, and their expression increases with worsening of the glomerulonephritic condition. Larger numbers of infiltrating NK cells are found in the glomeruli of diseased SLE-like MRL/MpJ and MRL/MpJ^{lpr} mice compared to healthy controls, concomitantly with the increase in NKG2D ligand expression.

6. Constitutive and localized expression of the NKG2D ligand Rae-1 increases the percent of mature CD11b^{High}CD27^{Low} and NK cell production of IFN-γ.

7. Preliminary results show the expression of the human NKG2D ligand MICA via immunohistochemistry in four out of six SLE patients with an active disease state.

Our studies using p85β deficient mice to characterize the role of p85β in NK cells and in NKG2D signaling demonstrate the following:

8. The deletion of the p85β subunit does not influence NK cell cellularity, differentiation, commitment, proliferation or conjugation.

9. The deletion of the p85β subunit marginally increases CD107a and cytotoxicity after NKG2D and non-NKG2D specific activation in IL-2-activated NK cells. The deletion of the p85β subunit increases cytokine secretion in IL-2-activated NK cells after NKG2D and NK1.1 activation

10. p85β deficient NK cells internalize less NKG2D after receptor activation, possibly leading to the an increased activatory state.

Conclusiones

Basándonos en los resultados obtenidos en los estudio sobre el papel de las células NK en la patogénesis del lupus utilizando modelos de ratón (MRL/MpJ y MRL/MpJ^{lpr}) que desarrollan una patología similar al lupus humano (SLE), podemos concluir que:

1. Los bazos de ratones MRL/MpJ que desarrollan lupus presentan una reducción en el porcentaje y el números absoluto de células NK, comparados con los bazos de ratones MRL/MpJ sanos. Esta reducción de células NK es similar a la que se observa en pacientes humanos con un estado activo de lupus.

2. Las células NK de los ratones MRL/MpJ y MRL/MpJ^{lpr} que desarrollan lupus presentan una disminución en los niveles de expresión de marcadores de maduración, siendo esta disminución mayor en el bazo que en la medula ósea.

3. Los ratones MRL/MpJ y MRL/MpJ^{lpr} que desarrollan lupus acumulan células NK inmaduras en periferia pero no en la médula ósea: Hay un aumento del número total de precursores de células NK y de las células NK inmaduras con el perfil CD11b^{Low}CD27^{High} en el bazo pero no en la médula ósea de ratones enfermos. Las células NK obtenidas de ratones MRL/MpJ^{lpr} enfermos y activadas con IL-15 muestran defectos en la degranulación (de la proteína CD107a) y en la capacidad de conjugarse con células dianas.

4. La mayor parte de las células NK que infiltran los riñones de ratones sanos y enfermos presentan un fenotipo de población madura CD11b^{High}CD27^{Low}. Las células NK que infiltran los riñones de ratones MRL/MpJ y MRL/MpJ^{lpr} que desarrollan lupus muestran un fenotipo más activado que el de las células NK que infiltran los riñones de ratones sanos control: Hay más células NK maduras CD11b^{High}CD27^{Low} en los riñones de ratones enfermos, y éstas producen más IFN-γ y presentan mayores niveles de fosforilación de STAT5 que las células NK en la periferia. Los mayores niveles del complejo IL-15/IL-15R detectados en los riñones de ratones enfermos podrían explicar el aumento del número células NK maduras infiltradas.

5. Los ligandos del receptor NKG2D, Rae-1 y Mult-1, se expresan de forma específica en los glomérulos de los ratones que desarrollan lupus. La expresión de estos ligandos aumenta con la progresión de la patología glomerulonefrítica. En los glomérulos de los ratones enfermos hay más infiltración de células NK que en los glomérulos de ratones control, lo que correlaciona con el aumento de la expresión de los ligandos NKG2D.

6. La expresión constitutiva y localizada del ligando de NKG2D Rae-1ε en modelos de ratón aumenta el porcentaje de la población NK madura $CD11b^{High}CD27^{Low}$ y la producción de IFN-γ por parte de células NK.

7. Resultados preliminares demuestran la expresión del ligando de NKG2D MICA en cuatro de seis pacientes con SLE con un estado activo de la enfermedad.

Los resultados obtenidos utilizando ratones deficientes en p85β para caracterizar el papel de la subunidad p85β de PI3K en el desarrollo y función de las células NK, así como en la señalización vía NKG2D nos permiten concluir que:

8. La ausencia de la subunidad p85β no influye en el numero, diferenciación, proliferación, o conjugación de células NK.

9. La ausencia de la subunidad p85β aumenta de forma marginal la degranulación (de la proteína CD107a) y la citotoxicidad de células NK cultivadas con IL-2 cuando éstas entran en contacto con células diana que expresan ligandos de NKG2D o de Ly49G2. La ausencia de la subunidad p85β permite una mayor secreción de citoquinas en células NK cultivadas con IL-2 cuando se activan a través de los receptores NKG2D y/o NK1.1.

10. Células NK deficientes en p85β internalizan menos receptor NKG2D tras su activación, lo que posiblemente conduce a un estado de activación mas agudo en estas células NK.

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Annex I

Compendium of manuscripts under revision or preparation obtained via the work described in the thesis

Under revision:

Spada R, Pérez-Yagüe S, Barber DF. Organ-specific phenotypic and functional properties of NK cells and NKG2D ligands in murine systemic lupus erythematosus models, 2013, Arthritis and Rheumatism – Manuscript ID: ar-13-0669

Manuscript in preparation

Spada R, Morillas L, Mejías R, Carrasco JM, Pérez-Yagüe S, Barber DF. The p85 beta subunit modulates NKG2D receptor internalization in murine NK cells

Spada, R, Barber DF. Natural Killer Cells in Systemic Lupus Erythematosus

Compendium of published articles achieved during this thesis

Published:

Zotes TM*, **Spada R***, Mulens V, Pérez-Yagüe S, Sorzano C, Okkenhaug K, Vanhaesebroeck B, Carrera AC, Barber DF. PI3K p110d is expressed in CD31+ endothelial splenic stromal cells and contributes to the segregation of T and B areas by regulating chemokine production, 2013, PLOS ONE
*these authors contributed equally to this work

Zotes TM, Arias CF, Fuster JJ, **Spada R**, Pérez-Yagüe S, Hirsch E, Wymann M, Carrera AC, Andrès V, Barber DF. PI3K p110g deletion attenuates murine atherosclerosis by reducing macrophage proliferation but not polarization or apoptosis in lesions, 2013, PLOS ONE

Mejías R, Pérez-Yagüe S, Gutiérrez L, Cabrera LI, **Spada R**, Acedo P, Serna CJ, Lázaro FJ, Villanueva A, Morales MdelP, Barber DF. Dimercaptosuccinic acid-coated magnetite nanoparticles for magnetically guided in vivo delivery of interferon gamma for cancer immunotherapy, 2011, Biomaterials